The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 51 APRIL, 1958 NO. 4

The Official Publication of the Central Society for Clinical Research

Clinical and Experimental

Rates of Appearance and Disappearance of White Blood Cells in Normal and in Various Disease States. Seymour Perry, M.D., Charles G. Craddock, Jr., M.D., and John S. Lawrence, M.D., Los Angeles, Calif. ---------- 501

Eosinophilic Response to Intraperitoneal Blood. John S. Chapman, M.D., and Rolland C. Reynolds, M.D., Dallas, Texas ---------- 516

Serum Macroglobulin Levels in Relation to Age, Sex, and Disease. Nils Eriksen, Ph.D., Seattle, Wash. ---------- 521

The Effect of Corticotrophin, Cortisone, and Prednisone on Whole Blood Coagulation Time. F. Dreyfuss, M.D., and J. Menczel, M.D., Jerusalem, Israel ---------- 530

Studies on Human Serum Gamma Globulin. II. Observations in a Case of Acquired Hemolytic Anemia Due to Cold Autoantibodies, in Multiple Myeloma, and in Other Clinical Problems. Alexander S. Wiener, M.D., Donald K. Briggs, M.D., Leo Weiner, M.D., and Lee Burnett, New York, N. Y. ---------- 539

The Influence of Peritoneal Puncture With and Without Intraperitoneal Air Insufflation on Renal Function and Electrolyte Excretion. Neal S. Bricker, M.D., and Lloyd Gregory, Jr., M.D., St. Louis, Mo., and Denver, Colo. ---------- 546

The Relationship Between Alveolar and Blood Carbon Monoxide Concentrations During Breathholding. Robert H. Jones, M.D., Marjorie F. Ellicott, M.D., A.M., John B. Cadigan, M.D., and Edward A. Gaensler, M.D., Boston, Mass. ---------- 553

The Differential Diagnosis of Biliary Tract Obstruction With Radioactive Rose Bengal. R. A. Nordyke, M.D., and W. H. Blahd, M.D., Los Angeles, Calif. With the Technical Assistance of Lucille Shoop, R.N., Billie Jean Kinney, and Marianne Lederer ---------- 565

The Effect of Hydrocortisone and Desoxycorticosterone on Experimental Infections With M. Tuberculosis in Rabbits. Richard L. Schultz, Ph.D., and Mabel S. Okawaki, M. S., Denver, Colo. With the Technical Assistance of Jack A. Moorman, Harold L. Seerveld, Jeanne LaCerte, and James S. Mullins ---------- 580

Objective Evaluation of Patients With Rheumatic Diseases. IV. Comparison of the Diphenylamine Reaction (DPA) With Serum Glycoprotein and Seromucoid Levels. M. R. Shetlar, Ph.D., and R. W. Payne, M.D., Oklahoma City, Okla. ---------- 588

Peripheral Biochemical Changes Associated With the Intravenous Administration of Ammonium Salts in Normal Subjects. Malcolm P. Tyor, M.D., and William P. Wilson, M.D., Durham, N. C. ---------- 592

(Contents continued on inside front cover)

PUBLISHED BY THE C. V. MOSBY COMPANY, ST. LOUIS 3, MISSOURI

Copyright © 1958 by The C. V. Mosby Company

Contents—*(Continued from front cover)*

The Hemolytic Effect of Primaquine. VIII. The Effect of Drug Administration on Parameters of Primaquine Sensitivity. C. L. Flanagan, M.D., Stanley L. Schrier, M.D., Paul E. Carson, M.D., and Alf S. Alving, M.D., Chicago, Ill. ---------- 600

Laboratory Methods

Determination of Bromide Space by Means of a Polarographic Method. Helen S. Jones, Ph.D., and Mary L. Terry, A.B., Boston, Mass. ---------- 609

Serum Protein Partition Analysis: Correlation Between Paper Electrophoretic and Turbidimetric Methods. D. R. Davis, B.S., F. A. Teague, B. J. Dietz, and R. E. Budd, Bethesda, Md. ---------- 616

Fluid Film Method of Electrophoresis. Newton Ressler, Ph.D., Norman A. Nelson, M.D., and Harold D. Oster, M.D., Eloise, Mich. ---------- 623

A Simple Nephelometric Test for Beta Lipoproteins of Human Serum. H. N. Antoniades, Ph.D., J. L. Tullis, M.D., L. H. Sargeant, M.D., R. B. Pennell, Ph.D., and J. L. Oncley, Ph.D., Boston, Mass. ---------- 630

A Modified Method for Evaluation of Clinical Usefulness of Antibiotics. Ilse Hoette, M.S., and Adrianus P. Struyk, Ph.D., Delft, Holland ---------- 638

Modification of the Tubeless Bedside Test for Gastric Acidity. J. M. Holthaus, M.D., F. L. Humoller, Ph.D., and J. R. Walsh, M.D., Omaha, Neb. ---------- 654

A Model Spotting Apparatus for the Automatic Deposition, on Filter Paper, of Solutions for Chromatographic Separations. John Logothetis, M.D., and Frederick Sherman, Minneapolis, Minn. ---------- 658

A Method of Holding Mice for Intravenous Injection. Ned Feder, M.D., Bethesda, Md. 662

Procedure for Increasing the Sensitivity of Tests for Specific Treponemal Antibody. Barbara J. Rosenau, Sc.D., and John F. Kent, Ph.D., Washington, D. C. With the Assistance of Robert W. Sanders and Lois A. Simonton, B.S. ---------- 664

(Editorial and Business Communications on page 44)

Editor
WILLIAM D. ROBINSON, M.D.

Associate Editor
WILLIAM H. BEIERWALTES, M.D.

University Hospital
University of Michigan Medical School
1313 Ann Street, Ann Arbor, Michigan

Board of Editors

FRANK H. BETHELL, M.D.
University of Michigan, Ann Arbor

RICHARD B. CAPPS, M.D.
University of Illinois, Chicago

WILLIAM H. DAUGHADAY, M.D.
Washington University, St. Louis

ELMER L. DeGOWIN, M.D.
State University of Iowa, Iowa City

RICHARD V. EBERT, M.D.
University of Arkansas, Little Rock

ROBERT A. GOOD, M.D.
University of Minnesota, Minneapolis

WENDELL H. HALL, M.D.
University of Minnesota, Minneapolis

RICHARD L. LANDAU, M.D.
University of Chicago, Chicago

OSCAR D. RATNOFF, M.D.
University Hospitals, Cleveland

DONALD W. SELDIN, M.D.
University of Texas, Dallas

WILLIAM A. SODEMAN, M.D.
Jefferson Medical College, Philadelphia

RICHARD W. VILTER, M.D.
University of Cincinnati, Cincinnati

Vol. 51, No. 4, April, 1958. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription rates: United States and its Possessions $13.00, Students $6.50; Canada, Latin-America, and Spain $13.50, Students $7.00; Other Countries $14.50, Students $8.00. Single copies $2.00 postpaid. Entered as Second Class Matter October 8, 1915, at the Post Office at St. Louis, Missouri, under Act of March 3, 1879. Printed in the U. S. A.

New DIFCO *Antisera*

BACTO-*C* PROTEIN ANTISERUM

for detection and quantitation of inflammatory diseases

★

BACTO-STREPTOCOCCAL ANTISERA

for detection of Streptococci of Groups A B C D F G

★

BACTO-*E. COLI OB* AND *O* ANTISERA

10 SEROTYPES and 2 POLYVALENT

for identification of Enteropathogenic E. Coli Serotypes

Specify DIFCO *Antisera*

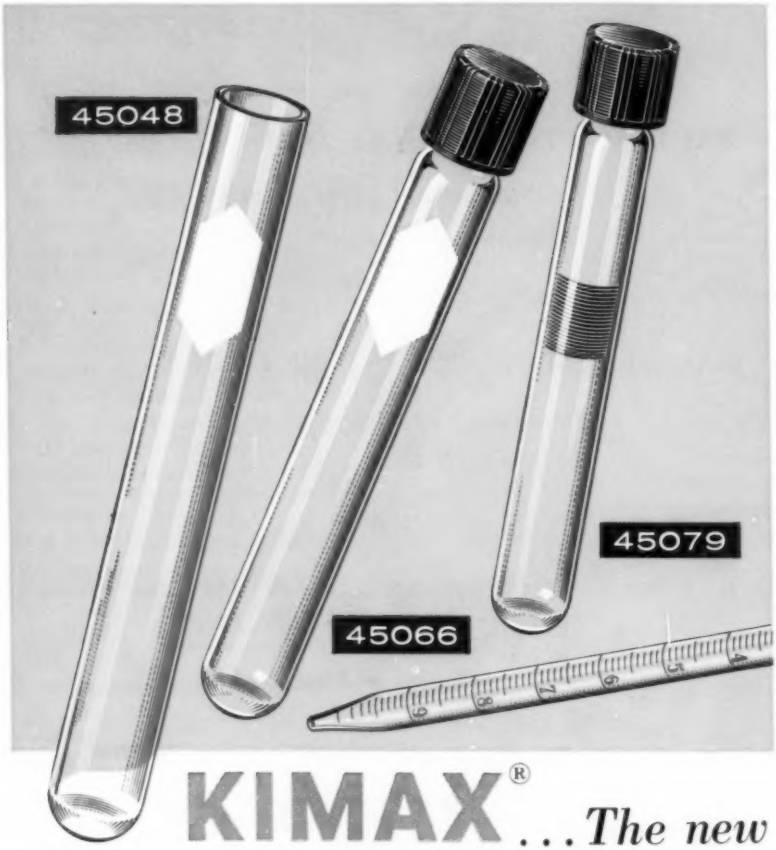
POTENT • SPECIFIC • RELIABLE

AVAILABLE ON REQUEST

Booklet 153—Bacto-*C* Protein Antiserum
Booklet 154—Bacto-*E. Coli OB* and *O* Antisera
Booklet 155—Bacto-Streptococcal Antisera

DIFCO LABORATORIES

DETROIT 1, MICHIGAN



KIMAX® ... *The new*

45048

CULTURE TUBE

Made of KG-33 "hard" glass for chemical durability and heat-shock resistance. Flat glazed tops. Large blasted marking area. Sizes from 6 x 50 to 38 x 200 mm.

45066

CULTURE TUBE (Screw cap)

Similar to 45048 but with top finished for screw cap with rubber or Teflon liner. Caps will withstand autoclaving and caps and liners are interchangeable.

45079 to 45084

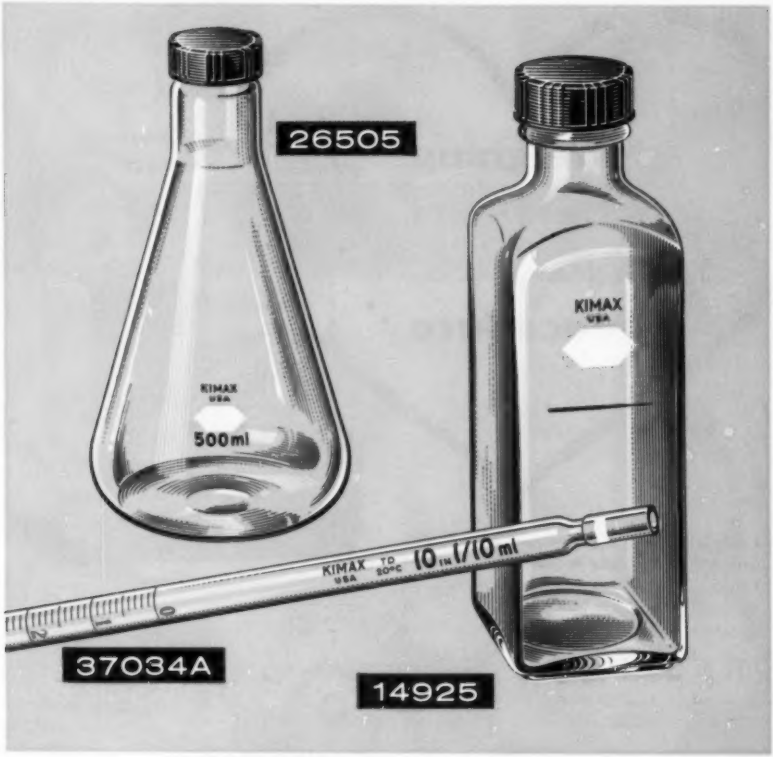
COLOR-CODED TUBES

Similar to 45066 but coded in six colors for quick, convenient identification. Fused-on enamel color patches are durable, withstand washing, sterilizing.

Now, Kimble builds new durability into laboratory glassware, thanks to KG-33 borosilicate glass.

KIMAX apparatus is highly resistant to thermal shock, mechanical shock and chemical attack.

Ask your Kimble dealer how you may qualify for quantity discounts. Kimble Glass Company is a subsidiary of Owens-Illinois, Toledo 1, Ohio.



standard of quality in "hard glass"

37034-A

PIPET

Constriction provides secure cotton plug seat. Tops are uniformly flat for better finger control. Tips gauged for accurate delivery. Sizes from 1/10 ml to 10 ml.

26505

ERLENMEYER FLASK

Specially designed by Kimble for use in mixing and storing culture media and for many chemical purposes. Caps can be steam sterilized.

14925

MILK DILUTION BOTTLE

Complies with requirements of APHA. Features smooth marking spot. Graduation line at 99 ml. Has plastic screw cap with cemented-in rubber liner.

KIMAX is available through dealers in the United States, Canada and principal foreign cities.

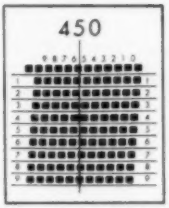
KIMBLE LABORATORY GLASSWARE
AN (O-I) PRODUCT

OWENS-ILLINOIS
GENERAL OFFICES • TOLEDO 1, OHIO

Weighing is the heart of all analytical procedure

To keep pace with increased laboratory work loads balance manufacturers have developed balances that weigh faster, are easier to operate and require *no operator technique*. The NEW Christian Becker Model NA-1 single pan, semi-automatic balance provides everything the chemist has wanted. Fast accurate weighings are as easy as 1 - 2 - 3.

1. Place sample on pan, close door, select "rough weighing" beam by rotating knob "A".
2. Weight control knobs "B" are then rotated. Weight indicator lights "C" *indicate if weights have to be added or removed.*
3. Final 1000 mgs., or any part thereof are recorded on new, large, easy to read reticle and vernier, after fine weighing beam is put in use. This ten thousand division optical readout "D" is absolutely unique. It eliminates completely "balance squint." The large number at the top plus the vertical line show that the weight is between .455 and .456 gram. By following the line down, it makes a solid rectangle at "4", thus giving a reading of .4554. This plus the weight recorded on counter "E", gives total weight of sample.



TEN THOUSAND DIVISION OPTICAL READOUT

New, large, easy to read reticle and vernier eliminates Balance Squint.

ANYONE CAN OPERATE THIS BALANCE

It also includes many other features:

- **External zero adjustment—temperature compensation**
- **Oil damping—longer "Pan-to Stirrup" dimension**
- **Exclusive two-beam construction—one for rough weighing—one for fine**
- **Weighing and pilot lights to indicate beam in use**
- **Stainless steel Class M weights**
- **Knobs for weights and beams are at convenient table height**
- **Weight counter, and reticle and vernier are at eye level**

Capacity 200 grams. Sensitivity .1 mg. Readability .05 mg. Catalog #H1830. Price $1,185.00
(4 page bulletin available on request)

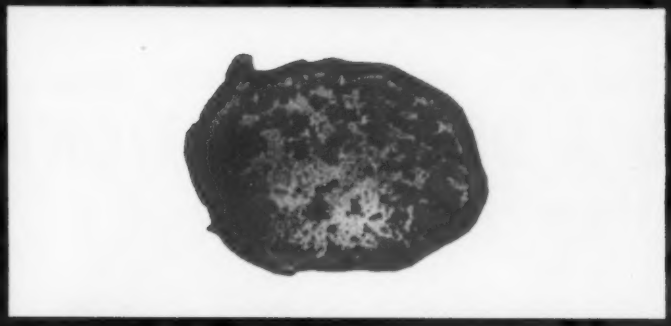
HARSHAW

HARSHAW SCIENTIFIC

Division of The Harshaw Chemical Company • Cleveland 6, Ohio

Sales Branches and Warehouses: **Cincinnati 13, Ohio • Cleveland 6, Ohio • Detroit 28, Michigan**
Houston 11, Texas • Los Angeles 22, California • Philadelphia 48, Pennsylvania

Sales Offices: **Baton Rouge, Louisiana • Oakland 11, California • Buffalo 2, New York**
Hastings-On-Hudson 6, New York • Pittsburgh 22, Pennsylvania



specific

Ortho Pharmaceutical Corporation
RARITAN, NEW JERSEY

Ortho

Before you buy your hematocrit centrifuge...

DEMAND A DEMONSTRATION!

International's

new **Quiet Test**

MODEL MB

RUNS QUIETER, YET COOLER...

than any previous hematocrit centrifuge.

- NEW "CONVERSATION PEACE" IN YOUR LAB... you can actually carry on a telephone conversation while the new Quiet Test is running nearby.
- PUSH-BUTTON ELECTRICAL BRAKE halts centrifuge in just 15 seconds!
- COOLER... than any previous hematocrit centrifuge.
- HIGH SPEED... up to 12,300 RPM.
- LONG LIFE... precision made with heavy duty motor, more rugged shaft and key components.

NEW PRECISION READER... MODEL CR—Fast, foolproof readings from any length capillary tube. The magnifying glass and light under tube make readings easier, faster and more accurate.

International Equipment Co.

1215 SOLDIERS FIELD ROAD, BOSTON 35, MASS.

Please have your representative call as soon as possible to arrange a demonstration.

Name..... Title.....

Institution.....

Street & No..... City & State.....

DESIGNED FOR THE **BUSY LABORATORY**

disposable

monoject®

"100"

THE ALUMINUM HUB NEEDLE

NOW PACKED IN A SEALED PLASTIC WRAPPER.
NON-PYROGENIC, SURGICALLY CLEAN AT NO EXTRA COST.

always CLEAN — always SHARP
DISPOSABLE to ELIMINATE the
danger of CROSS-INFECTION.

The efficiency and economy of **monoject "100"** has been tested and proven by America's leading hospitals. ***Costs less than 4¢ a needle.***

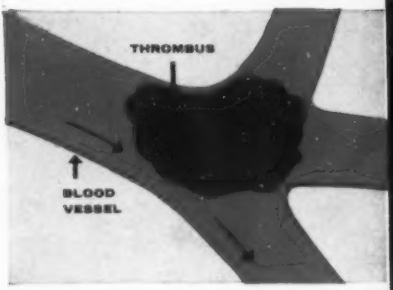
MANUFACTURED IN U.S.A. BY

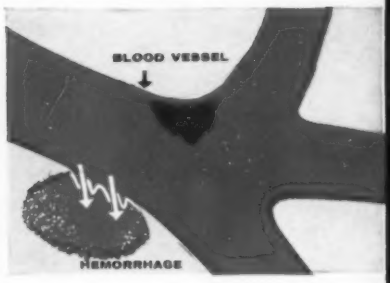
ROEHR PRODUCTS CO., Inc.

WATERBURY, CONN. • DELAND, FLA.

World's Largest Manufacturer of Hypodermic Needles

write ROEHR PRODUCTS CO., Inc., DeLand, Fla *for further information*





too little and too much in anticoagulant therapy

"Thromboembolic diseases may be treated or recurrences prevented through the use of anticoagulant drugs which reduce the clotting power of the blood, so that thrombi, if present, remain small and harmless and new clots do not form.

However, these drugs are potentially dangerous to use; too little and the thrombus grows in spite of the treatment, too much and the patient may have a spontaneous hemorrhage."*

Simplastin—the pre-extracted thromboplastin-calcium—is specifically and uniquely controlled to give you accurate reproducible results in the therapeutic range. This is the major factor in helping the clinician avoid the dangers of too little or too much.

Available from leading laboratory supply distributors in boxes of ten 6-determination and 20-determination vials.

Simplastin®

maximum accuracy
minimum work

*Excerpt from the teaching film strip (35 mm with 33⅓ r.p.m. record) "Determining Prothrombin Activity" —available at no charge from: Laboratory Supply Division, Film Department, WARNER-CHILCOTT, Morris Plains, N. J.

Although not yet using Simplastin, I've heard much about it from my colleagues. Without obligation on my part, please send me three 20-determination vials of Simplastin.

Name_____

Laboratory_____

Address_____

City_____

State_____

☐ Please furnish name of nearest distributor.

Laboratory Supply Division
WARNER-CHILCOTT
MORRIS PLAINS, N. J.

Simplicity...
Dependability

Transaminase Kits

by DADE

Accurate colorimetric method for determination of SGO-T and SGP-T

Transaminase Kits provide reliable and reproducible results. Each kit contains reagents for 100 tests. Calibration standard is in liquid form and maintains its stability indefinitely. Conveniently packaged so reagents may be kept together when not in use.

SIMPLE PROCEDURE FOR DETERMINATION OF SGO-T AND SGP-T

Add 0.2 ml of serum to prepared buffered substrate and incubate in 37°C. water bath for one hour. (Only 30 minutes required for SGP-T.) Add color developer. Allow to stand at room temperature for 20 minutes. Add 10 ml 0.4N NaOH to tube and allow to stand for 5 minutes. (Sodium hydroxide not supplied with kit.) Read in any laboratory colorimeter at 490 to 510 mμ wavelength.

No. 53951—Transaminase Kit for SGO-T, complete with Aspartic-Keto substrate, DNPH color developer and calibration standard $12.50

No. 53952—Transaminase Kit for SGP-T, complete with Alanine-Keto substrate, DNPH color developer and calibration standard $12.50

Scientific Products

DIVISION OF AMERICAN HOSPITAL SUPPLY CORPORATION

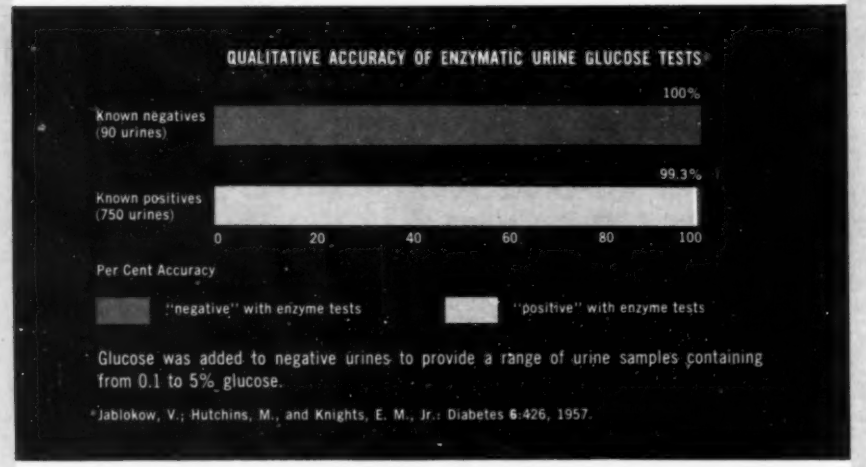
GENERAL OFFICES— 1210 LEON PLACE, EVANSTON, ILLINOIS

Division Offices: Atlanta • Chicago • Columbus • Dallas • Kansas City • Los Angeles • Minneapolis New York • San Francisco • Washington

AN AMES CLINIQUICK
TECHNICAL BRIEFS FOR THE CLINICAL LABORATORY

what is the role of the enzymatic urine glucose test in the laboratory?

It is a rapid, efficient screening procedure. The enzyme test was proved qualitatively accurate—positive or negative for urine glucose—in more than 1900 tests. All positive results are checked with an accepted quantitative test.*



Glucose was added to negative urines to provide a range of urine samples containing from 0.1 to 5% glucose.

*Jablokow, V.; Hutchins, M., and Knights, E. M., Jr.: Diabetes 6:426, 1957.

"yes-or-no" specific enzyme test for urine glucose

CLINISTIX® BRAND Reagent Strips

- rapid screening procedure
- just "dip-and-read"
- sharp color change
- firm strip for easy handling

for reliable quantitative estimations

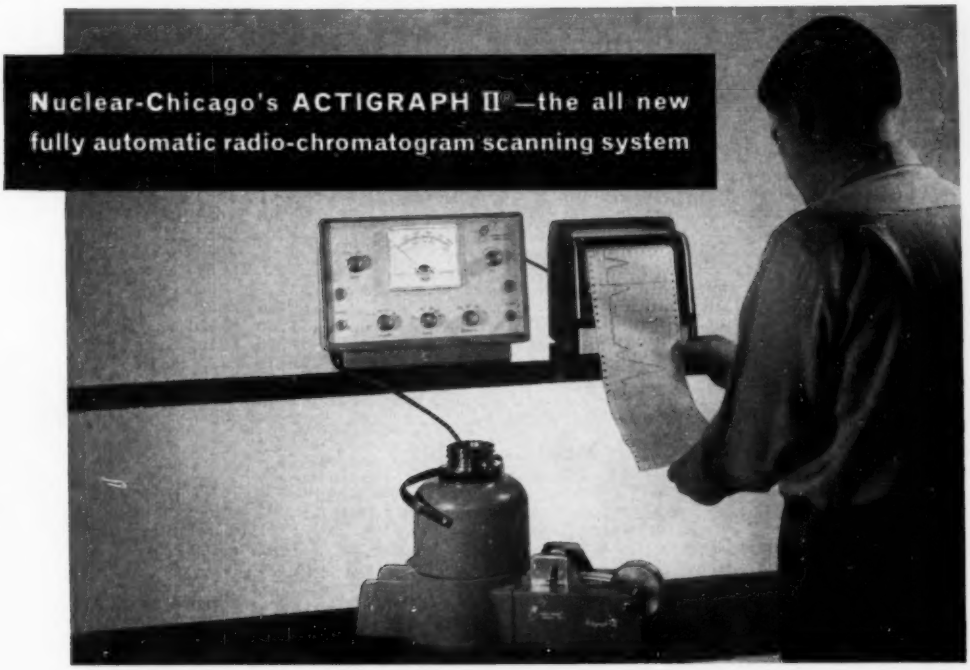
CLINITEST® BRAND Reagent Tablets

familiar blue-to-orange spectrum... standardized "plus" reporting... optimal sensitivity ... reliable readings throughout the critical range

AMES COMPANY, INC · ELKHART, INDIANA
Ames Company of Canada, Ltd., Toronto

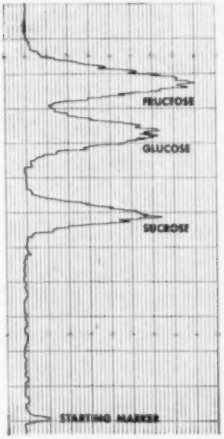
46658

a new radioisotope counting system for biological and clinical research



Completed chart can be placed next to strip to directly determine location and intensity of radioactive zones.

The new Actigraph II is a completely redesigned and improved scanning system for recording automatically the position and total activity of separated radioactive zones on a strip chromatogram. Two reels accommodate strips up to 50 feet in length allowing single short strips to be spliced end-to-end for continuous operation without attention. A wide choice of detectors may now be used including G-M tubes, *windowless gas flow counters*, and scintillation detectors. Increased sensitivity is provided through improved geometry. The Actigraph II is available with a wide variety of chart recorders including the new "rectilinear" recorder. Chart and strip speeds are easily synchronized by making identical gear changes. For complete information write us and ask for "Nucleus #14".



Typical radio-chromatogram scan made with Actigraph II

Fine Instruments - Research Quality Radiochemicals

nuclear - chicago
CORPORATION
249 WEST ERIE STREET • CHICAGO 10, ILLINOIS

Paragon Reagents • Dependable • Tested • Paramount Quality

Paragon Standards and Reagents for Clinical Chemistry

Accurately prepared and standardized in our own laboratories.

Description	Catalog No.	Quantity	Price
Acid Digestion Mixture—Paragon (concentrated)	P745	100 cc	$1.20
Alkaline Copper Tartrate—Paragon (concentrated)	P851	for 1 liter	.45
Ammonium Sulfate Solution—Paragon (saturated-aqueous)	P582	100 cc	1.00
Benedict Compound Paragon—Qualitative, granules	P520	for 1 gallon	1.35
	P501	for 1 liter	.45
Benedict Compound Paragon—Quantitative, granules	P631	for 1 gallon	2.70
	P611	for 1 liter	.90
Paragon Bone Decalcifying Solution	PS3100	500 cc	2.35
Benzoic Acid Solution—Paragon (0.25% aqueous)	P890	500 cc	1.00
Paragon Combined Decolorizer-Counterstain	PS2275	500 cc	1.80
Creatinine Standard—Paragon (stock—1 ml. equals 1 mg.)	P360	100 cc	.75
Creatinine Standard—Paragon (working—1 ml. equals .006 mg.)	P370	100 cc	.75
Diazo Reagent A—Paragon (0.1% sulfanilic acid)	P575	250 cc	.80
Diazo Reagent B—Paragon (0.5% sodium nitrite)	P576	100 cc	.75
Ferric Ammonium Sulfate—Paragon (ferric alum powdered)	P676	50 gm.	1.45
Ferric Chloride Solution—Paragon (10% aqueous)	P970	250 cc	.90
Glucose Standard—Paragon (stock—1%)	P870	100 cc	1.00
Glucose Standard—Paragon (intermediate—stock—0.2%)	P872	100 cc	1.00
Glucose Standard—Paragon (1 ml. equals 0.1 mg.)	P875	100 cc	1.00
Glucose Standard—Paragon (1 ml. equals 0.2 mg.)	P876	100 cc	1.00
Icteric Index Standard—Paragon (0.01% potassium bichromate)	P590	250 cc	.85
Nessler Compound Paragon, granules	P701	for 1 liter	1.90
Nitrogen Standard—Paragon (stock—1 ml. equals 1 mg.)	P722	100 cc	.85
Nitrogen Standard—Paragon (working—1 ml. equals 0.1 mg.)	P723	100 cc	.85
Nitrogen Standard—Paragon (working—1 ml. equals 0.05 mg.)	P724	100 cc	.85
Nitrogen Standard—Paragon (stock—1 ml. equals 0.2 mg.)	P725	100 cc	.85
Nitrogen Standard—Paragon (stock—1 ml. equals 1.5 mg. in 0.1N sulfuric acid)	P726	100 cc	1.00
Nitrogen Standard—Paragon (working—1 ml. equals 0.015 mg. in 0.01N sulfuric acid)	P727	100 cc	1.00
Nitrogen Standard—Paragon (stock—1 ml. equals 0.75 mg.)	P728	100 cc	.85
Nitrogen Standard—Paragon (working—1 ml. equals 0.015 mg.)	P729	100 cc	1.00
Phenolphthalein Solution—Paragon (1% alcoholic)	P960	100 cc	.85
Phospho-Molybdic Acid Reagent—Paragon, concentrated	P801	for 1 liter	2.35
Picric Acid Solution—Paragon (especially purified—saturated—aqueous)	P350	500 cc	1.50
Standard Potassium Thiocyanate Solution—Paragon (1 ml. equals 1 mg. chlorine)	P651	250 cc	1.00
Standard Potassium Thiocyanate Solution—Paragon (1 ml. equals 1 mg. sodium chloride)	P656	250 cc	1.00
Standard Silver Nitrate Solution—Paragon (1 ml. equals 1 mg. chlorine)	P650	250 cc	.75
Standard Silver Nitrate Solution—Paragon (1 ml. equals 1 mg. sodium chloride)	P655	250 cc	.75
Sodium Alizarin Sulfonate Solution—Paragon (1% aqueous)	P965	100 cc	.60
Sodium Hydroxide Solution—Paragon (10%)	P355	500 cc	1.50
Sodium Hydroxide Solution—Paragon (0.1N)	P950	500 cc	1.20
Sodium Tungstate Solution—Paragon (10%)	P116	500 cc	2.25
Sulfuric Acid—Paragon (2/3N)	P120	500 cc	1.20
Sulfuric Acid—Paragon (1/12N)	P125	500 cc	1.20
Toepfer's Reagent—Paragon (0.5% alcoholic)	P955	100 cc	1.00
Van den Bergh Standard—Paragon (cobalt sulfate)	P580	250 cc	1.50

Write for catalog #54 fully describing Paragon Staining Solutions, Chemical Reagents and Solutions and Paragon Tray Drawer Cabinets and accessories

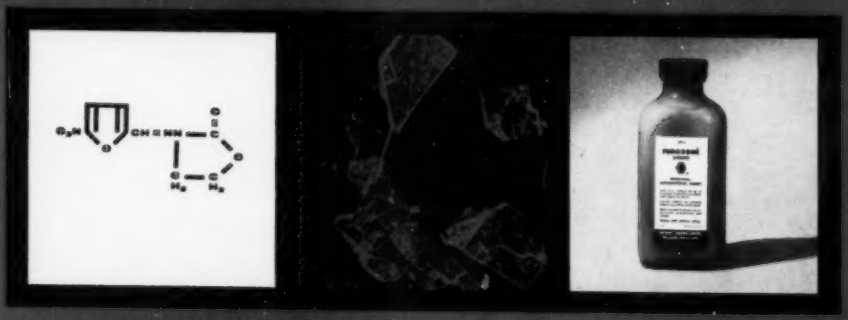
All prices F.O.B. New York, N. Y., subject to change without notice.

Manufactured exclusively by

PARAGON C. & C. CO., INC., 2540 Belmont Ave., New York 58, New York

Cable Address: Wijeno, New York

new wide-range nitrofuran
controls the "problem pathogens" of
bacterial diarrheas and enteritis



FUROXONE®

brand of furazolidone

LIQUID

antibacterial
demulcent
adsorptive

A finely divided suspension containing Furoxone, 50 mg. per 15 cc., with kaolin and pectin ■ Pleasant orange-mint flavor

■ For patients of all ages (may be mixed with infant formulae; passes through a standard nursing nipple).

■ Perorally effective against a wide range of enteric bacteria[1-3]—including common pathogenic species and strains of Escherichia, Salmonella and Staphylococcus not adequately controlled by antibiotics and sulfonamides.

■ Bactericidal rather than bacteriostatic.

■ Does not induce development of significant bacterial resistance, nor predispose to monilial or staphylococcal overgrowth.

■ No toxicity reported.[1]
Mild sensitization, nausea, emesis occur occasionally.

Supplied in bottles of 240 cc.
Also available: FUROXONE Tablets, 100 mg. scored, bottles of 20 and 100.

1. Ponce de Leon, E.: Antibiotic Med. & Clin. Therapy 4:816, 1957.
2. H. W. McFadden and M. M. Musselman: Personal communication to Eaton Laboratories.

NITROFURANS A unique class of antimicrobials Products of Eaton Research

Eaton Laboratories, Norwich, New York

2
specialized isotope instruments...
now combined in 1 compact system
NEW AUTOMATIC SPECTRA-SCALER
Now you can increase the accuracy, speed and scope of your isotope studies with this automatic scaler-and spectrometer combination by Reed-Curtis Nuclear Division. The SPECTRA-SCALER performs the specialized functions of a separate pulse height analyzer and an automatic scaler, and provides a built-in high voltage supply incorporating a silicon rectifier for longer life...all in a single compact cabinet that measures just 9½" x 15" x 19¼".
*SPECTRA-SCALER APPLICATIONS...
• Blood Volume (with well counter) (with assembly pictured at right)
• I-131 Thyroid Uptake
• Liver Function
• Kidney Function
New operational features include a push-button computing circuit to simplify comparison of patient's radioactivity measurement with a standard, by direct reading in percent...electrical reset timer and register...and both preset counter and preset timer, built-in and fully automatic.
Tuning resolution is six times faster than any other instrument.
Write for medical instrument catalog to:
AMERICAN ELECTRONICS, INC.
REED-CURTIS NUCLEAR DIVISION
655 West Washington Blvd., Los Angeles 15, Calif.

For Faster Specimen Preparation
Complete / Compact / Convenient

ALOE PRECISION Micro Section Grinding and Polishing Apparatus

Facilitates preparation of undecalcified specimens of bone, metal, and other materials for microscopic and radiographic examination.

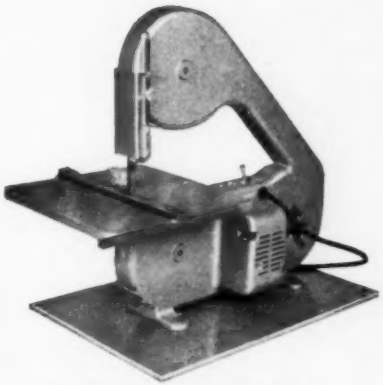
Without extensive pretreatment, you can obtain sections ranging from 10 to 25 microns in thickness, using this technic.

Procedure: Specimens are first cut with a special, low-speed band saw; defatted in acetone for a short period; then placed in molds containing liquid plastic and allowed to set overnight at room temperature.

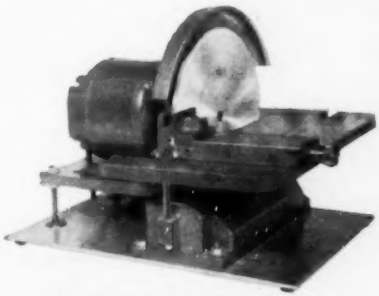
Resulting blocks are then cut with band saw to 1 mm sections and glued to object holders. Removable brass stops on object holder determine final thickness of specimen. Each side of section is applied to abrasive wheel until desired thickness is achieved. For thinner or a more highly polished section, specimen block is rubbed over a glass polishing plate several times.

All instruments are small enough to fit inside an average size hood, or other enclosure, when used with radioactive or noxious materials.

Write Today for Prices and Complete Information.



60200—Micro-Specimen Electric Band Saw



60210—Micro-Specimen Electric Grinder-Polisher

Typical Specimen Blocks Showing Sample Sections

aloe

ALOE SCIENTIFIC

DIVISION OF A.S. ALOE COMPANY

5655 Kingsbury, St. Louis 12, Mo.

14 Fully Stocked Divisions Coast-To-Coast

Another packaging improvement from **B&A®**

Introducing

genetron® PLASTIC CAPS *for Fuming Acids*

Replace ground-glass stoppers and plaster-of-Paris seals on such hard-to-handle reagents as Fuming Sulfuric and Fuming Nitric... simplify opening, handling

Typical of Baker & Adamson's continuing packaging developments is this new screw-cap closure for fuming sulfuric acid, fuming nitric acid and other hard-to-handle reagents. It links research in two fields—reagent packaging and fluorine chemistry—to make your laboratory work simpler and easier.

The new screw-caps are made of General Chemical's "Genetron" Plastic HL (high molecular weight polymer of trifluorochloroethylene)—a material with extreme chemical and thermal resistance. As a result, it is possible to eliminate the old-fashioned glass stoppers and plaster-of-Paris seals traditionally used with these reagents. Also, the new bottles have pour-clean lips—giving you greater handling and pouring control.

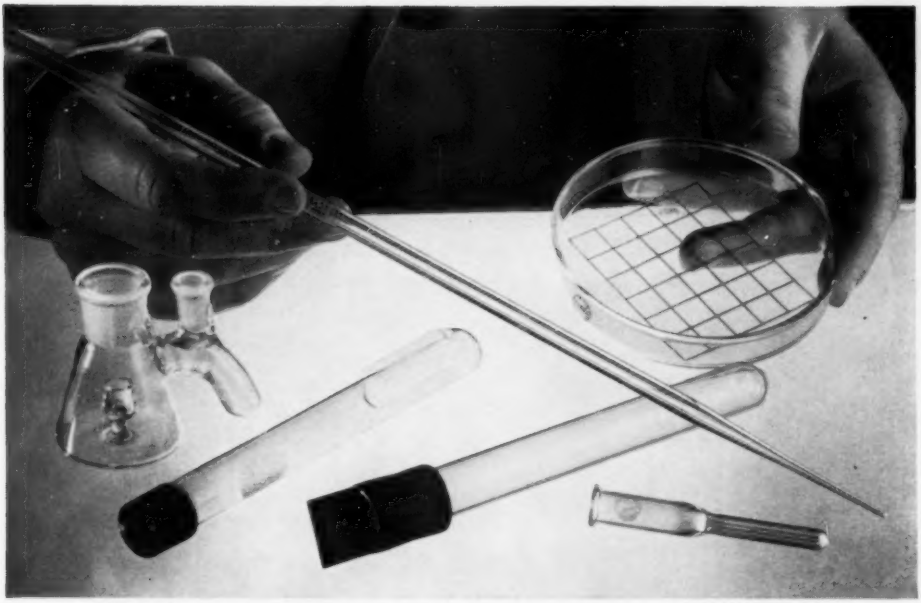
When ordering reagents, be sure of the *latest* and *best* in packaging, as in purity. Always specify B&A.

BAKER & ADAMSON®
Reagents

GENERAL CHEMICAL DIVISION
ALLIED CHEMICAL & DYE CORPORATION
40 Rector Street, New York 6, N. Y.

Offices: Albany* • Atlanta* • Baltimore* • Birmingham* • Boston* • Bridgeport* • Buffalo* Charlotte* • Chicago* • Cleveland* (Ohio) • Denver* • Detroit* • Houston* • Jacksonville Kalamazoo • Los Angeles* • Milwaukee* • Minneapolis • New York • Philadelphia* • Pittsburgh* Portland (Ore.) • Providence • St. Louis* • San Francisco* • Seattle • Kennewick* • Vancouver* and Yakima (Wash.) **In Canada: The Nichols Chemical Company, Limited • Montreal* • Toronto* • Vancouver***

**Complete stocks carried here.*



(Left to right) Serological Pipette, Long tip, double bevel ground for strength (#7084); Warburg Flask, Single side arm (#5530); Tissue Culture Tube, with flat window, screw cap (#9831); Culture Tube, Non-toxic rubber snap cap (#9821); Centrifuge Tube, Micro, Plain with cylindrical bottom (#8090); Petri Dish, LIFETIME RED grid on exterior of bottom (#3165).

485 *NEW* PYREX® items plus all the other labware you need are listed in this new catalog

What you see in the picture is just part of the assortment of *new* labware, created by Corning for you.

The 485 new items join some 9,000 others—all fully detailed in Catalog LG-1. And, for the first time, you'll find specifications on *both* standard and custom ware between the covers of a *single* volume.

There are 348 pages, *color-coded* so you can quickly locate and identify what you need.

You get the new items, plus the PYREX lab glassware you're already familiar with, from your regular lab supply dealer.

The catalog you get from us, FREE. Just fill in the coupon and send it on its way. We'll do the rest.

CORNING GLASS WORKS
82-4 Crystal Street, Corning, New York

Please send me the NEW, complete Laboratory Glassware Catalog, LG-1.

Name.....

Title.....

Company.....

Street.....

City.....

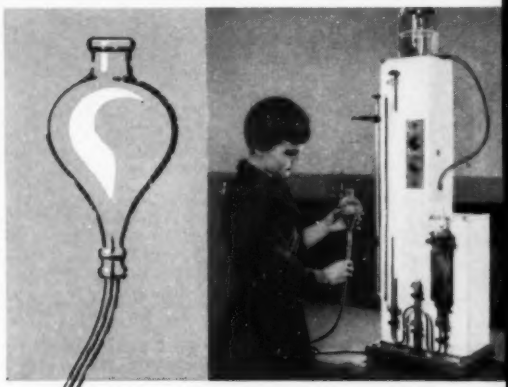
Zone.....State.....

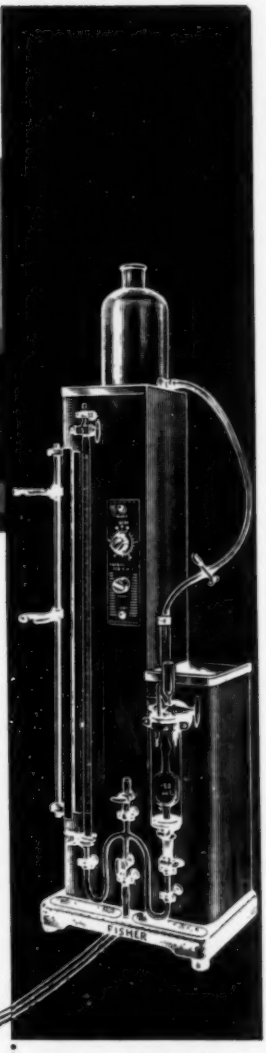
Corning means research in Glass

PYREX® laboratory ware

. . . the tested tool of modern research

FISHER HAS IMPROVED
THE BASIC APPARATUS
FOR GAS
IN BLOOD OR PLASMA





the new

FISHER-VAN SLYKE

manometric apparatus

the price 330^{00}

The completely modernized Fisher-Van Slyke Manometric Blood-Gas Apparatus is more efficient and accurate than ever. Carbon monoxide, carbon dioxide, oxygen and other components in blood, serum and other body fluids can be expelled and measured from very small samples.

Clinical chemists recognize the Manometric Apparatus as the most accurate for any reaction that *uses up* or *evolves* a gas.

A few of the many measuring applications—

- Carbon Dioxide
- Carbon Monoxide
- Oxygen
- Nitrogen
- Hydrogen
- Ammonia
- Amines
- Sugars
- Hemoglobin
- Urea
- Lactic Acid
- Methemoglobin
- Iodates
- Calcium
- Potassium

FISHER SCIENTIFIC

IN THE U.S.A. Boston, Buffalo, Charleston, W.Va., Chicago, Cleveland, Detroit, New York, Philadelphia, Pittsburgh, St. Louis, Washington **IN CANADA** Edmonton, Montreal, Toronto

America's Largest Manufacturer-Distributor of Laboratory Appliances & Reagent Chemicals

Write for Bulletin FS-271

A 12-page bulletin describes applications and advantages of this and other Fisher-Van Slyke analytical apparatus.

120 Fisher Building
Pittsburgh 19, Pa.

D-73b

fine
reagent
specialties . . .
HARLECO
HARTMAN-LEDDON COMPANY
PHILADELPHIA PENNSYLVANIA
Catalog upon request
AVAILABLE OF REPRESENTATIVE SUPPLY HOUSES

X-RAY TECHNOLOGY

by

CHARLES A. JACOBI, Head, X-Ray Technology, Oregon Technical Institute, Oretech, Oregon, and **DONALD E. HAGEN,** Instructor, X-Ray Technology, Oregon Technical Institute, Oretech, Oregon. With Foreword by **JAMES M. HILTON, M.D.,** Roentgenologist and Course Supervisor, X-Ray Technology, Oregon Technical Institute.

410 pages, 320 illustrations. Price, $9.75.

Presents basic information and procedures to assist technicians on their own in the smaller hospitals and clinics and technicians of limited x-ray experience.

Believing that the x-ray technician should understand the general principles of the working of the machine he operates the authors have included chapters on the principles of physics and electricity. In the chapters on positioning the authors related anatomy and physiology to the positioning because they believe that a complete knowledge of osteology and a substantial acquaintance with the remaining anatomy and physiology are the bases for developing a skillful, proficient technician.

Written by practicing x-ray technicians it contains all of the necessary fundamentals, plus detailed information as to positioning. Even trouble shooting is covered.

Positioning occupies a major portion of the book. Here are shown the most effective angles and positions for x-raying all areas of the body—the extremities, trunk, skull, and viscera. Preceding each of the major areas are chapters on anatomy and physiology related to such areas.

THE C. V. MOSBY COMPANY
3207 Washington Blvd.
St. Louis 3, Missouri

Date_____

Gentlemen: Send me Jacobi-Hagen "X-RAY TECHNOLOGY," priced at $9.75.

☐ Attached is my check. ☐ Charge my account.

Name _____ Street _____

City _____ Zone _____ State _____

Lab-4-58

Pre-cleaned—in the patented *Stand-Rite* ™ dispenser box

Gold Seal Slides, long the *Standard of Quality* in leading laboratories, now bring you still more handling ease and convenience than ever. The secret: the new patented Stand-Rite box with simple push-in tabs which keep slides upright as you use them! The result is fast, fumble-proof handling; no fingermarks; ready-to-use slides. Gold Seal Slides, made from crystal clear, uniform glass, are non-corrosive and selected to eliminate all imperfections. They will not fog, even after years of use.

Dispensed clean from new lint-free plastic box holding one ounce

New, modern Gold Seal packaging means clean cover glasses...added convenience and protection. Quickly and cleanly removed—one at a time—cover glasses may be used right from the box for many applications. Red snap-open box supports glasses between walls of white plastic; lintless foam blanket cushions them when box is closed. Made of rigidly specified non-corrosive, non-fogging glass of uniform thinness. Gold Seal Cover Glasses are individually inspected, *guaranteed perfect.*

Ask your dealer about the complete range of sizes in which Gold Seal Micro Slides and Cover Glasses are available.

Clay-Adams
NEW YORK 10

"RBC" and HEMOGLOBIN DETERMINATIONS.... *IN SECONDS!*

with

The NEW and PROVEN MODEL "G" Haemoscope

MORE RAPID
MORE ACCURATE
MORE DEPENDABLE
MORE REPRODUCIBLE

Incorporates Many New Features

The new MODEL G HAEMOSCOPE is a precision instrument for rapid, accurate and reproducible RBC and Hemoglobin determinations. The Haemoscope functions as a scatter photometer, measuring the light scattered directly—not at right angles to the light source—by particles in suspension. It is capable of compensating for variations in cell size due to the highly precise optical bench and method of selecting a discrete portion of total scattered light pattern.

Sahli type pipettes are used to collect blood samples and standard volumetric pipettes or burettes are used to prepare the diluents. Gower's solution is employed as the RBC diluent while a very dilute ammonium hydroxide solution is used for the Hb tests. Three simple controls are quickly mastered. Repeated settings are eliminated and only one dial is to be read. Operator can achieve reproducible and accurate results to within ½% of each other, using the same sample, without previous experience or training.

Both RBC and Hb results are read directly from one dial, the RBC to the nearest 25,000 cells and the Hb to the nearest one-tenth of one gram. The MODEL G uses permanent, all-glass standards which permit quick and simple check-out of the unit.

Handsomely finished in "eye-ease" green, acid-resistant enamel. Dials and controls are clearly engraved with widely spaced scale divisions. Operates on 115 or 220 volts, AC current only.

Cat. No. M-52085 HAEMOSCOPE, Model G—including operating manual and glassware kit consisting of:
1 ea. Bulb; 2 ea. Haemoscope, Cuvette; 4 ea. Vial, Shell, RBC Dilution; 4 ea. Vial, Shell, Hb Dilution; 6 ea. RBC and Hb Combination Pipette; 1 ea. Volumetric RBC Dilution Pipette; 1 ea. Volumetric Hb Dilution Pipette. *Please specify voltage, frequency and type of current when ordering* **$615.00**

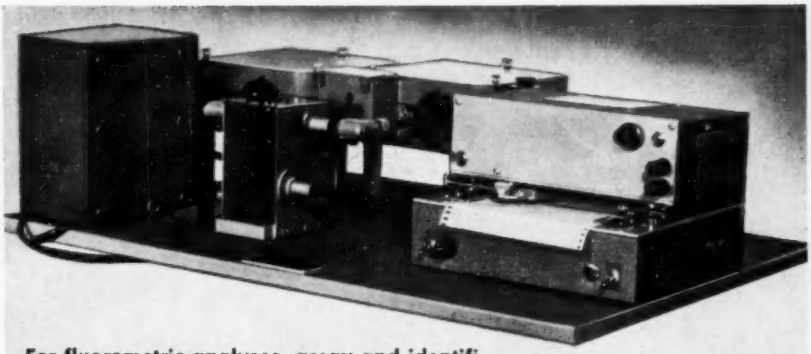
Cat. No. M-52085A HAEMOSCOPE, Model G—without glassware kit **$590.00**

ORDER TODAY!

STANDARD SCIENTIFIC SUPPLY CORP. • 1888 •

STANDARD SCIENTIFIC
Supply Corp. 808 BROADWAY NEW YORK 3, N.Y.

LABORATORY APPARATUS
REAGENTS AND CHEMICALS



For fluorometric analyses, assay and identification of chemical constituents... in the ULTRAVIOLET, VISIBLE and INFRARED REGIONS

THE FARRAND®

Spectrofluorometer

FOR MANUAL OPERATION, FOR RECORDING OR FOR OSCILLOSCOPE PRESENTATION

The Farrand Spectrofluorometer is designed for determining the optimum wavelength for activating Organic Compounds and the optimum wavelength for measuring their emitted fluorescence spectra.

The instrument is simple to operate, and the efficiency of the optical and detector system affords great sensitivity and response even when using capillary or micro volumes of highly diluted solutions.

It is not of a console-type construction, and therefore can be serviced readily without dismantling.

BULLETIN NO. 820 UPON REQUEST

FOCI TRADE MARK

FARRAND OPTICAL CO. INC.

Bronx Blvd. and E. 238th St., New York 70, N. Y.

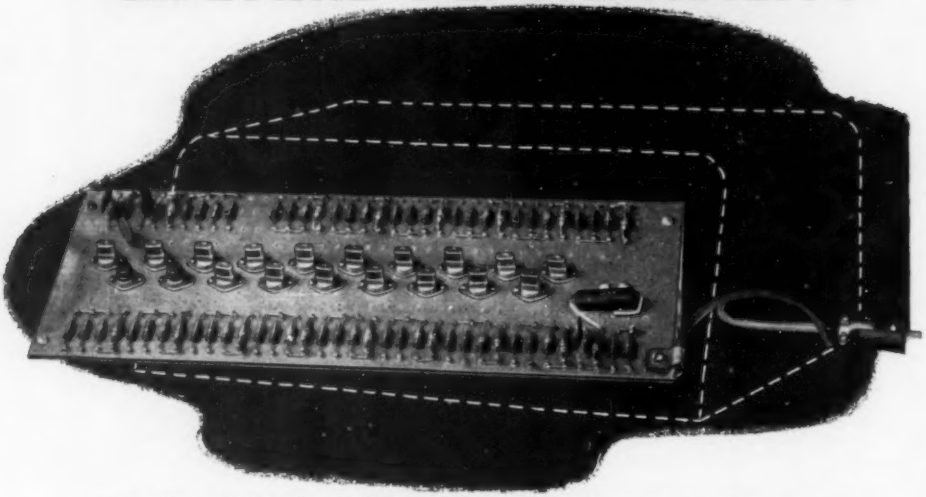
Engineering Research • Development • Design • Manufacture

Precision Optics, Electronic and Scientific Instruments

NOW.. NRD SCALERS and COUNTING CIRCUITS

"TRANSISTORS MAKE THE DIFFERENCE"

with **LIFETIME DEPENDABILITY**



The all new NRD TRANSISTORIZED scalers and counting circuits offer research accuracy with lifetime dependability. "Transistorizing" assures trouble-free operation because they replace troublesome vacuum tubes. They also eliminate heating problems . . . permit more compact design . . . greater portability . . . with lower power requirements.

What are your needs? Write today for new catalog on NRD *transistorized* instruments.

NRD INSTRUMENT COMPANY

9842 MANCHESTER RD. • ST. LOUIS 19, MO.

a DIVISION of

nuclear corporation of america, inc.

NUCOR

add confidence

to your cross match

ANTI-HUMAN SERUM

FOR THE COOMBS ANTI-GLOBULIN TEST

Ortho Pharmaceutical Corporation

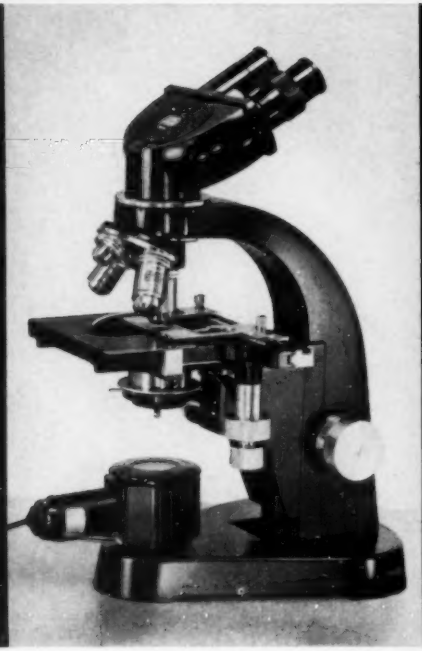
RARITAN, NEW JERSEY

Ortho

A NEW CONCEPT IN MICROSCOPE PERFORMANCE

Leitz LABOLUX

LABOLUX S 47/92-15 inclined binocular microscope, with built-in mechanical stage #47; Abbe condenser; quadruple nosepiece with achromats 3.5x, 10x, 45x and 100x oil immersion, the last two having spring-loaded mounts; paired 6x and 10x binocular eyepieces; with carrying case $696.50



LABOLUX for photomicrography with LEICA.

Combined binocular-monocular tube.

The Leitz LABOLUX medical and laboratory microscope is a scientifically engineered instrument of modern design, built for a lifetime of use. The LABOLUX is a new concept in fatigue-free operation and precision performance. It combines the coarse and fine focusing adjustments in a single control; with all controls, including the actuating knob for the mechanical stage, in a low convenient position. High power lenses have spring-loaded mounts preventing damage to lenses or slides.

The LABOLUX can be faced away from the observer, for increased accessibility to all controls and to the object stage. Interchangeable body tubes permit binocular or monocular observation as well as photomicrography, with simultaneous observation and photography made possible through a unique trinocular attachment. A wide variety of accessories makes the Leitz LABOLUX the ideal instrument for hospital or office laboratory.

E. LEITZ, INC., DEPT. L-4
468 Fourth Ave., New York 16, N.Y.

Please send me the Leitz LABOLUX brochure

Name_____

Street_____

City_____ Zone_____ State_____

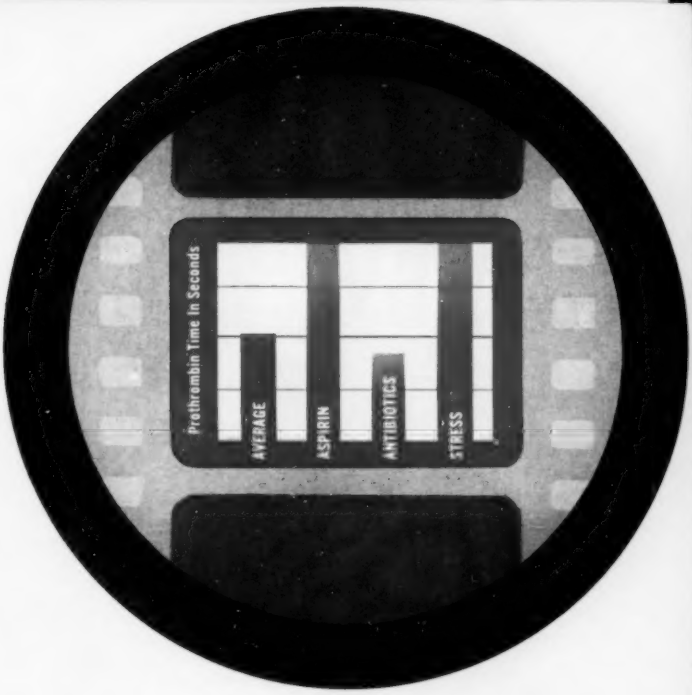
Leitz

FIRST IN PRECISION OPTICS

20987

E. LEITZ, INC., 468 FOURTH AVENUE, NEW YORK 16, N.Y.
Distributors of the world-famous products of Ernst Leitz G.m.b.H. Wetzlar, Germany-Ernst Leitz Canada Ltd.
LEICA CAMERAS · LENSES · MICROSCOPES · BINOCULARS

a prothrombin control: the blood of a patient?



"Considerable variations from the normal can be produced by high concentration of aspirin, by therapeutic amounts of antibiotics or by general stress. For this reason, a control obtained from a single individual should not be used.

"The blood of a patient can never serve as a control because the effect of stress, pain, anxiety, worry or recent anesthesia may affect the prothrombin time of his blood."*

Diagnostic Plasma Warner-Chilcott is pooled human plasma which is further standardized by the addition of isolated clotting factors to give constant prothrombin times. To use Diagnostic Plasma, one need only add distilled water.

Diagnostic Plasma consistently gives results identical with those of pooled human plasma, assuring accurately controlled anticoagulant dosage.

Diagnostic Plasma

WARNER-CHILCOTT

Although not yet using Diagnostic Plasma, I've heard much about it from my colleagues. Without obligation on my part, please send me three vials of Diagnostic Plasma Warner-Chilcott.

Name _____

Laboratory _____

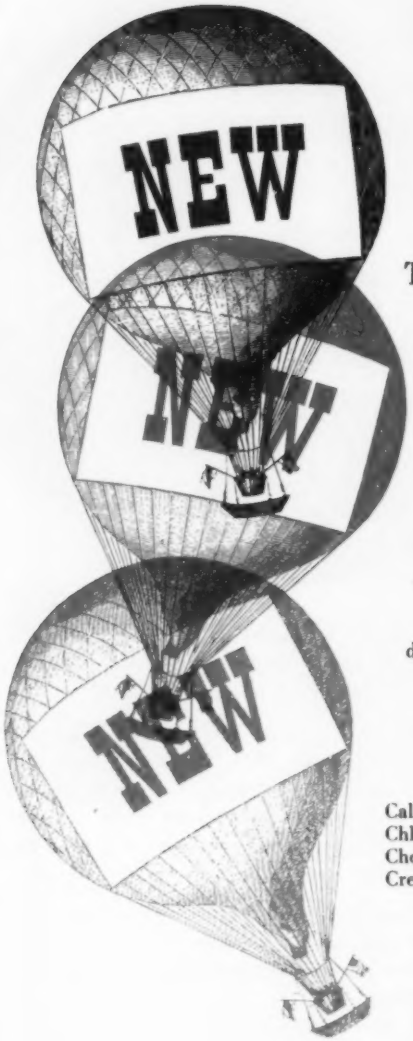
Address _____

City _____ State _____

☐ Please furnish name of nearest distributor.

Laboratory Supply Division
WARNER-CHILCOTT
MORRIS PLAINS N J

**Excerpt from the teaching film strip (35 mm with 33⅓ r.p.m. record) "Determining Prothrombin Activity" —available at no charge from: Laboratory Supply Division, Film Department, WARNER-CHILCOTT, Morris Plains, N. J.*



TRANSAMINASE DETERMINATION

(serum glutamic oxalacetic transaminase)

Now included in

HYLAND CLINICAL

CHEMISTRY CONTROL SERUM

Prepared only from freshly drawn human blood, Hyland Clinical Chemistry Control Serum provides the ideal control, since it is a *natural* reference standard with essentially the same composition as specimens under test. When run in parallel with your determinations on unknowns, there is nothing more dependable as a day-by-day check on instruments, reagents and techniques.

To assure accuracy, each production lot is assayed by three independent laboratories. Mean values and *acceptable range* for 13 components listed below (those found to be consistently reproducible and stable) are charted for each lot.

Calcium	Non-Protein Nitrogen	Sodium
Chloride	Phosphorus, inorganic	Total Protein
Cholesterol	Potassium	Transaminase (SGO-T)
Creatinine	Protein-bound Iodine	Urea Nitrogen
		Uric Acid

Simply add distilled water to prepare Hyland control standard for use. Reconstituted standard remains stable for *five days* when stored under refrigeration.

For "No Guesswork" Blood Chemistry Determinations —Specify Hyland Clinical Chemistry Control Serum (dried). Available in 5 cc. vials, 6 vials per box.

HYLAND

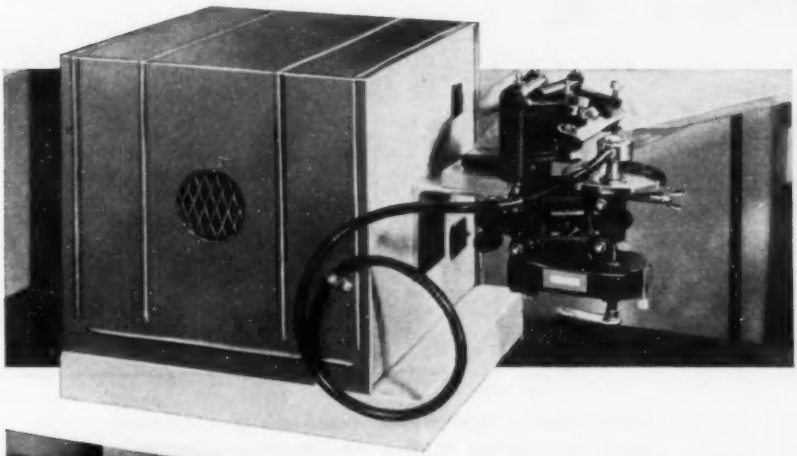
HYLAND LABORATORIES

4501 Colorado Boulevard, Los Angeles 39, California

252 Hawthorne Avenue, Yonkers, New York

Quiet, compact unit for tissue freezing–

S/P Histo-Freeze



New, portable freezing unit for greater convenience in the pathology laboratory. Histo-Freeze maintains uniform rate of cooling. Compact unit occupies only 12" x 18" area; no noisy or cumbersome CO_2 tanks needed.

Fits all popular makes of microtomes; simple to install—microtome is easily secured to plate on front of Histo-Freeze for safe, vibrationless operation. Circulation of Freon 12 through unit holds temperature at approximately −30°F. Just turn compressor standby switch on in the morning and unit is ready for use any time during the day. In standby operation power consumption is just 50 watts. Lightweight—unit weighs only 50 lbs.

	Each
No. 67027A—Histo-Freeze, table model tissue freezing unit, complete, for use with AO freezing microtome. 115 volts, 60 cycles AC.	$450.00
No. 67027B—Same but for Bausch & Lomb microtome.	
No. 67027C—Same but for Sartorius microtome.	

Scientific Products—*a single source for all laboratory supplies and equipment. For additional information, write our nearest Division office or consult your SP representative.*

Scientific Products

DIVISION OF AMERICAN HOSPITAL SUPPLY CORPORATION

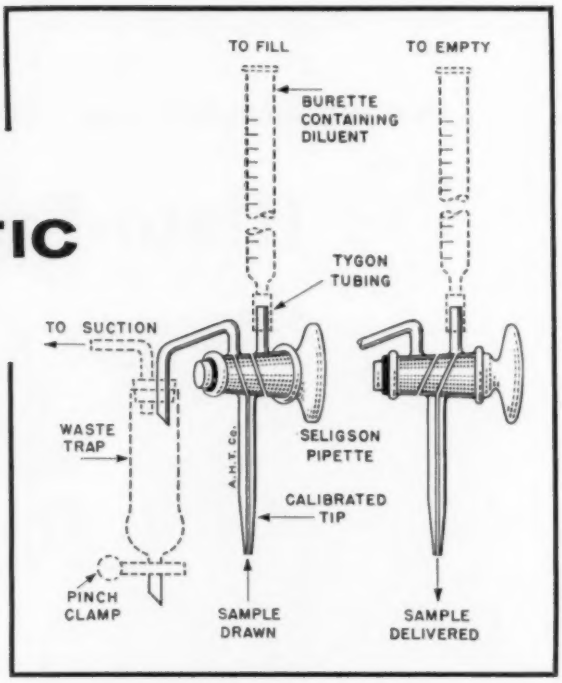
GENERAL OFFICES—1210 LEON PLACE, EVANSTON, ILLINOIS

Division Offices: Atlanta • Chicago • Columbus • Dallas • Kansas City • Los Angeles • Minneapolis

New York • San Francisco • Washington

Seligson AUTOMATIC PIPETTE*

- **SAVES**
  - Time
  - Glassware
- **REPRODUCIBLE**
  - Sampling
  - Delivery
  - Dilution
- **SELF CLEANING**



. . . eliminates individual sampling pipettes and repeated filling of dilution pipettes

A fixed volume pipette permanently attached to a double bore stopcock, which permits rapid filling without adjustment to a reference mark, and complete washout delivery of the contained sample by a measured amount of a second liquid from a burette, pipette, or arbitrarily calibrated tube connected to upper arm. Eliminates need for individual sampling pipettes, and repeated filling of dilution pipettes, realizing a highly significant economy of time and glassware, and assuring reproducible sampling, delivery, and dilution which are especially important in routine clinical chemistry. Applicable, however, to any type analysis in which a standard volume is diluted or reacted directly with a measured volume of a second liquid.

In use, the pipette is attached to the dispensing burette by means of the upright arm of the stopcock, and to a suction device by means of the bent tube at the left. A waste trap is inserted into the line prior to the suction connection.

Samples are taken by setting stopcock to connect tip with suction line. A small excess is drawn over into the bent tube to serve as an initial rinse for the pipette wall. The sample is delivered by reversal of the stopcock plug to allow solution in the burette to expel the sample through the tip, and to rinse it clean. Following delivery of the required amount of reagent from the burette, the tip, filled with diluent which keeps it clean, is ready for immediate intake of the next sample.

The pipette, devised by Dr. David Seligson of the Graduate Hospital of the University of Pennsylvania, has been applied to more than twenty-five clinical chemistry procedures as described in *American Journal of Clinical Pathology, Vol. 28, No. 2 (August, 1957) pp. 200-207,* and *Technical Bulletin of the Registry of Medical Technologists, Vol. 27, No. 7 (July, 1957) pp. 152-159.*

The pipettes are designed for attachment to standard burettes or to similar dispensing vessels. Connection is best made with Tygon tubing, which makes a transparent, flexible joint.

8209-B. Pipettes, Seligson, as above described.

To contain, ml.....	0.05	0.1	0.2	0.5	1.0
Tolerance, ml (±)...	0.005	0.005	0.01	0.01	0.05
Tip length, mm.....	65	55	65	160	140
Each.....	**11.00**	**11.00**	**11.00**	**11.00**	**11.00**

*Patent applied for.

ARTHUR H. THOMAS COMPANY

More and more laboratories rely on Thomas / *Laboratory Apparatus and Reagents*

VINE ST. AT 3RD • PHILADELPHIA 5, PA.

The Journal of Laboratory and Clinical Medicine

VOL. 51 APRIL, 1958 NO. 4

CLINICAL AND EXPERIMENTAL

RATES OF APPEARANCE AND DISAPPEARANCE OF WHITE BLOOD CELLS IN NORMAL AND IN VARIOUS DISEASE STATES

SEYMOUR PERRY, M.D., CHARLES G. CRADDOCK, JR., M.D., AND JOHN S. LAWRENCE, M.D.

LOS ANGELES, CALIF.

FOR over 30 years a great deal of effort has been expended in attempts to establish the lifespan of the leukocyte. Results have varied from less than 1 hour to 3 weeks, depending on the particular technique employed.[1] One of the earliest observations was that of Minot and Isaacs[2] who, in 1925, transfused blood from a patient with chronic lymphocytic leukemia into a patient with lymphosarcoma. The recipient's blood showed an elevation of lymphocyte count for 35 minutes before returning to normal. In 1945, Lawrence, Ervin, and Wetrich[3] cross-circulated leukocytes from a normal cat into an irradiated leukopenic cat. They observed a survival time for blood leukocytes of 16 to 24 hours. Weisberger and associates[4] found that the white counts of rats injected with rat lymphocytes tagged with P^{32} returned to normal in a matter of minutes. Recently, White[5] transfused atabrine-tagged white cells and found that these cells disappeared from the recipient's blood in 30 to 90 minutes.

It is quite obvious that all of these experimental techniques are subject to certain criticisms. Animal and human experiments involving cross-transfusion into normal or irradiated recipients raise the possibility of genetic or immunologic incompatibilities. The rate of utilization and the disappearance of white cells in irradiated animals may be quite different from that in the normal. In vitro labeling experiments, followed by subsequent administration

From the Department of Medicine, School of Medicine, University of California Medical Center and Wadsworth Veterans Administration Center, Los Angeles, Calif.

Supported in part by the U. S. Public Health Service, Parke, Davis & Company, and The Gladys F. Bowyer Fund.

Received for publication Aug. 26, 1957.

of the tagged cells to another animal, must result in damage to the cells used. Finally, these experiments determine only the length of time spent by the leukocyte in the vascular system which is no indication of their total survival time.

Recently, however, there have been several studies which have attempted to avoid these criticisms. Advantage has been taken of the fact that radioactive phosphorus (P^{32}) is incorporated into leukocyte desoxyribose nucleic acid (DNA) only during the intermitotic phase and is not exchanged subsequent to cell division.[6-10] It has been assumed that, in the dose used, the isotope does not affect cell physiology. With this technique Kline and Cliffton[1] concluded that, in the human, white cells were confined to the marrow for 4 days and then were released into the circulation, where they remained for another 9 days. Ottesen[11] calculated the mean age of granulocytes in the peripheral blood to be 8.7 and 9.4 days. Osgood,[6] using similar techniques, estimated the lifespan of granulocytes in chronic myelocytic leukemia to be about 3 days, and of lymphocytes in chronic lymphocytic leukemia about 30 days. Doering and his co-workers[12] observed the appearance of maximum leukocyte DNA-P^{32} activity to be 7 to 9 days after P^{32} administration in acute leukemia, 9 to 11 days in chronic myelogenous leukemia, and 14 to 15 days in chronic lymphocytic leukemia.

Employing this technique of leukocyte DNA-P^{32} incorporation, we have studied the rate of appearance in the blood and the intravascular lifespan of white blood cells in normal individuals and in patients with various diseases.

METHODS

The rate of appearance of DNA-P^{32}-labeled leukocytes was studied in a group of 21 patients including 4 hematologically normal individuals. Patients with the following clinical disorders were investigated: one with acute leukemia, one patient with chronic monocytic (Naegeli) leukemia, two with chronic myelocytic leukemia, 4 patients with chronic lymphocytic leukemia, one patient with myeloid metaplasia and a leukemoid reaction, one reticulum cell sarcoma and a leukemoid reaction, one with an acute infection, 2 with chronic infections, and 4 with polycythemia rubra vera. Two hundred and fifty or 500 μc of P^{32} as $NaHPO_4$ was given to all of the patients, with a few exceptions. No attempt was made to administer this on a weight basis. One patient with chronic myelocytic leukemia received 5 and 8 mc. of P^{32} on separate occasions. One patient with polycythemia rubra vera received 1 and 2 mc. at different times. The normal patients were all elderly individuals without hematologic disorders.

The patients were bled for leukocyte DNA-P^{32} determinations at appropriate intervals after administration of the isotope. It was necessary to obtain 150 ml. of blood if the leukocyte count was 10,000 per c.mm.[3] Smaller quantities of blood were required with higher counts. Siliconed glassware was used at all times, and the entire procedure was carried out at 4° C. Red and white blood cell counts, differential counts, packed cell volumes, and platelet counts were obtained simultaneously. White blood cells were separated with the aid of dextran and subsequent procedures were carried out in a cold room at 4° C. The leukocyte rich supernatant obtained with the dextran sedimentation was spun at 800 to 1,000 r.p.m. in an International Type 2 centrifuge for 10 to 15 minutes. This supernate was discarded. The white cell button was now washed with 0.85 per cent saline repeatedly for 5 minutes at 3,500 r.p.m. until clear supernate was obtained. The cells were then transferred into a graduate and diluted up to a specific volume

(i.e., 20 to 40 ml.), and multiple white blood cell counts were done on this suspension. After centrifugation, the white cells were washed with 1 per cent citric acid several times until the supernate became clear. The cells were then homogenized for 5 minutes in 1 per cent citric acid, and were washed with 0.2 per cent citric acid until the supernate cleared. After a final washing in cold saline, the cells were resuspended in saline so that approximately 0.1×10^{10} cells were present in 10 ml.

DNA-P^{32} extraction was accomplished using a method developed by Dr. Norman Simmons of the U.C.L.A. Atomic Energy Project. The purity of the DNA so obtained has been demonstrated in a previous publication.[13] The cell suspension obtained as above was placed in a beaker and an equal volume of 60 per cent sodium xylene sulfonate,* pH 7, was added with constant stirring. After further stirring for at least 15 minutes, the suspension was allowed to remain in the refrigerator overnight or longer. The Naxonate mixture was then stirred for at least 30 minutes or until the gel was thoroughly disrupted.

Two volumes of buffered acetate were added to the Naxonate mixture with stirring and the pH adjusted to 3.8 with 5 N acetic acid. After this had remained in the cold for 15 minutes it was centrifuged at 3,500 r.p.m. (or faster) for 15 minutes. The pH of the supernate was brought to 7.0 with 5 N NH_4OH and 3 to 5 Gm. celite No. 535 added with mixing. The mixture was filtered through a coarse sintered glass filter until a clear suspension was obtained. A 0.54 volume of absolute isopropyl alcohol was then added with constant stirring. The white precipitate (DNA) so obtained was dried with ethanol and redissolved in water.

Details of the P^{32} counting have been published previously.[13] All radioactivity was corrected to day 0, the day of P^{32} administration. Counting was continued for 5,120 counts on all samples which were done in duplicate, using a Nuclear-Chicago binary scaler, Model 161A, and a TGC—No. 2 Geiger-Müller tube with a statistical reproducibility of ± 1 per cent.

Methyl green-pyronine nucleic acid stains were done on some of the patients by a method recently described.[14]

RESULTS

Most of the patients showed no significant changes in peripheral white and red cell or differential counts during the course of study. Two patients with polycythemia rubra vera and one patient with chronic myelocytic leukemia receiving therapeutic doses of P^{32} had marked changes in their counts. Also, the patient with the acute infection responded to specific antibacterial therapy with a marked drop in his leukocyte count. Only the normal patients demonstrated a fall in packed cell volume and hemoglobin, as a result of the repeated venisections needed to obtain the leukocyte suspensions. Since the white blood counts in these individuals were normal, the amount of blood necessary to obtain a satisfactory yield of white cells had to be large in relation to the amounts needed in the other patients. However, the fall in hemoglobin was moderate; the lowest value obtained at the end of the study in any of the normal individuals was 10.5 Gm. per cent.

The rate of appearance of labeled leukocytes in the normal patients was remarkably consistent. Radioactivity was negligible for the first 2 or 3 days, but by the fourth day the rise in specific activity had accelerated. Peak specific activity was attained in all the normal patients by the eighth day when there were 9 or 10 c.p.m. per microgram DNA-P (c.p.m./γ-DNA-P) above background.

*Naxonate; G-Wyandotte, Las Nietos, Calif.

However, the decline in specific activity was much more gradual, so that one month after P^{32} administration it was approximately equal to that found on the sixth day. All leukocyte DNA-P^{32} activity usually disappeared between the fortieth and sixtieth days. Fig. 1 demonstrates the average leukocyte DNA-P^{32} in 4 normal patients.

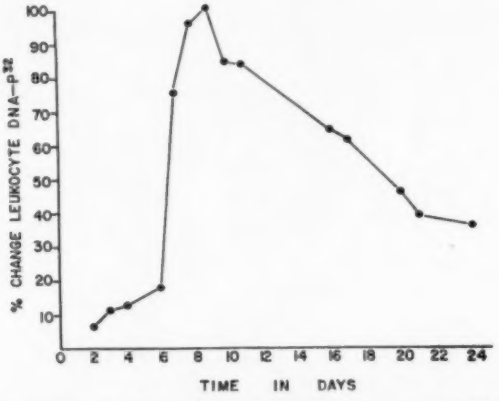


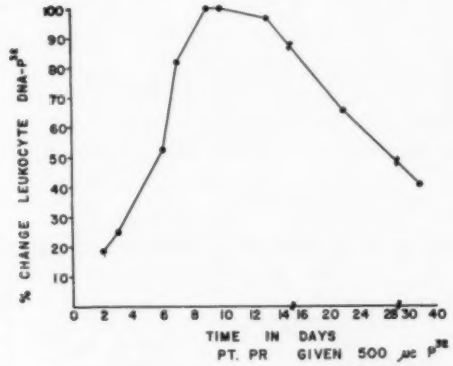
Fig. 1.—Appearance of P^{32}-labeled leukocytes in normal individuals. Four normal elderly patients were given 500 μc of P^{32}. Activity rises rapidly from the sixth day to reach a peak on the ninth day and then gradually falls.

No attempt was made in these experiments to separate the various types of white blood cells and analyze their individual contribution to this curve. From data obtained in dogs and in patients with chronic lymphocytic leukemia it is apparent that the specific activity contributed by DNA-P^{32} in lymphocytes is ordinarily very low as compared to the neutrophil. This observation agrees well with those of others.[8, 11] Therefore, the rapid increment and peak specific activity consistently observed after 6 days is believed to represent the specific activity of granulocytic cells as they are released from the marrow. As will be described subsequently, the appearance of DNA-labeled lymphocytes in the circulation occurs within a matter of a few hours after administration of P^{32} to dogs. It is probable that the low levels of specific activity of DNA-P^{32} observed in the first 6 days after giving the isotope represents the contribution of lymphocytes, as shown by Ottesen.[11]

One patient with myeloid metaplasia and a leukemoid reaction had a white blood count varying between 150,000 and 180,000 per c.mm. with immature myeloid forms ranging from 28 to 38 per cent. When he was studied with P^{32}, labeled cells appeared more rapidly than in the normal individuals with the peak of specific activity on the ninth and tenth days. The rate of decline did not differ from the normal but unfortunately it was not possible to follow this individual until all of the leukocyte DNA-P^{32} activity had disappeared. The results obtained in this patient are shown in Fig. 2. Peak specific activity represented 6 c.p.m. per microgram DNA-P above background.

In contrast to this patient with myeloid metaplasia and a leukemoid reaction is one with a leukemoid response secondary to a severe infection superimposed on reticulum cell sarcoma. In the latter case, labeled leukocytes appeared very early and disappeared rapidly from the peripheral blood (Fig. 3). Peak activity was 5.4 c.p.m. per microgram DNA-P. The peripheral white blood count, with antibiotic therapy, fell from 86,000 per c.mm. to 28,000 per c.mm. during the period of isotope study. There were no marked changes in the differential count, segmented neutrophils ranging from 83 to 91 per cent.

Fig. 2.



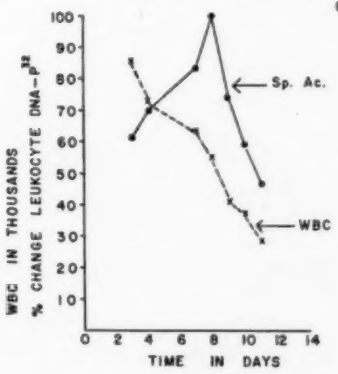


Fig. 3.

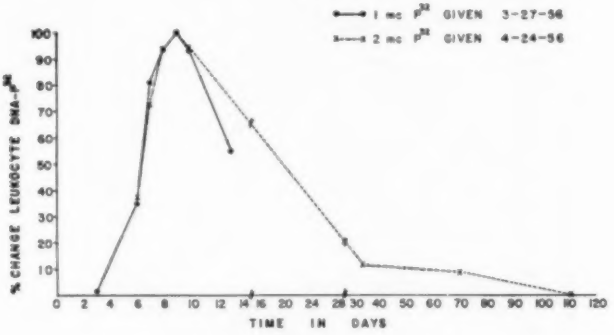
Fig. 2.—Specific activity of leukocyte DNA obtained in a patient with "burned out" polycythemia, myeloid metaplasia, and a leukemoid reaction.

Fig. 3.—A leukemoid reaction secondary to a severe infection in a patient with reticulum cell sarcoma. Note the rapid appearance of labeled cells and the falling white blood count.

In the patients with polycythemia rubra vera, the greatest activity was obtained on the ninth day. The rate of appearance of labeled cells appeared quite similar to that seen in the normal patients. The decline rate was perhaps slightly more rapid although insufficient observations have been made to establish this. Leukocyte DNA-P^{32} activity was still present 70 days after administration

of the isotope. The results in one patient (G) studied on two separate occasions are shown in Fig. 4. The curves are similar despite the difference in dosage of P^{32}. One hundred per cent activity represents 14 and 28 c.p.m. per microgram DNA-P background for the 1 and 2 mc. dosage, respectively. There was no significant change in either the white or red blood count during the period of study in this patient.

Fig. 4.



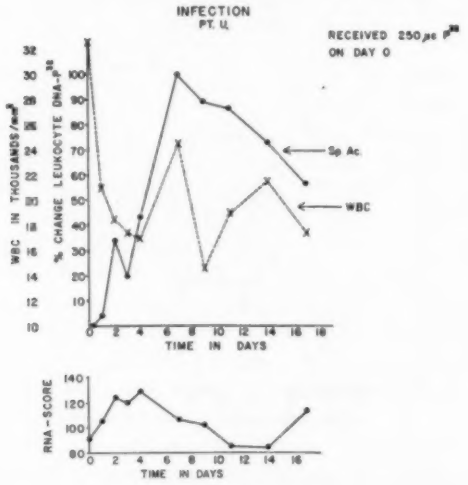


Fig. 5.

Fig. 4.—A patient with polycythemia rubra vera studied on two occasions with different doses of P^{32}. There were no significant changes in either the red or white blood count.

Fig. 5.—Appearance and disappearance of labeled white cells in a patient with an acute infection. Note the rise in the number of granulocytes containing ribose nucleic acid ("RNA score") coincident with the appearance of labeled white cells.

A patient with an acute bacterial bronchopneumonia was given a tracer dose of P^{32} 3 days after the onset of his illness. The rate of appearance of labeled leukocytes is shown in Fig. 5. Specific antibacterial therapy was started

simultaneously with administration of the isotope. Clinical improvement was prompt and the white blood count fell from 33,000 per c.mm.[3] with 81 per cent segmented neutrophils to 15,000 with 64 per cent neutrophils in 17 days. Labeled cells appeared early after the P^{32} injection, a peak being reached on the seventh day with 7.3 c.p.m. per γ-DNA-P above background. The RNA "score" was well above the average normal (88) and was coincident with the appearance of the labeled cells. This is a further indication that these cells are newly formed and are not merely "old" cells released from storage upon demand. The rate of disappearance of labeled cells did not appear to differ from that observed in normal individuals.

Two patients with chronic bronchiectasis were studied. Results in one of these is shown in Fig. 6. One hundred per cent activity was 5.5 c.p.m. per γ-DNA-P. Labeled white cells did not seem to appear more rapidly than in the normal. RNA "scores" were consistently high. Again, the rate of decline in leukocyte DNA specific activity was no different from the normal.

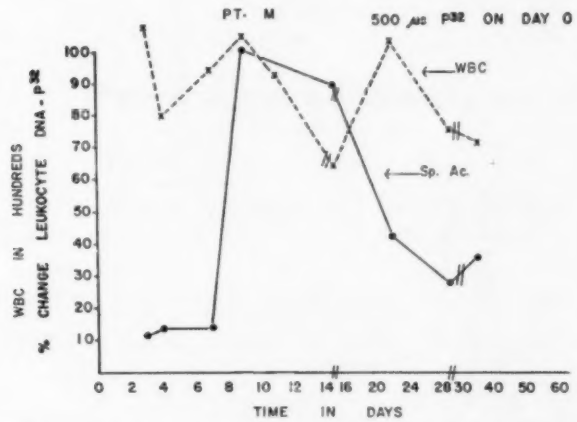


Fig. 6.—P^{32} specific activity of leukocyte DNA in a patient with bronchiectasis. The general shape of the curve appears normal.

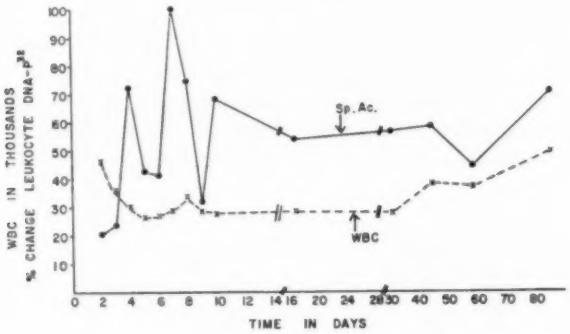
Results obtained in patients with chronic lymphocytic leukemia were remarkable in two respects. Specific activity demonstrated several peaks in all the patients studied. There was a rapid rate of appearance of labeled cells, two of the patients showing a peak on the second and third days. Another peak was present in all patients on the sixth or seventh day. Thereafter, occasional peaks occurred at different intervals. With one exception, all had DNA-P^{32} activity for over 80 days. In the one exception, all activity had disappeared between 30 and 60 days after P^{32} administration.

In general, the specific activity of lymphocyte DNA-P^{32} in the patients with chronic lymphocytic leukemia was extremely low in comparison to that in patients with other diseases studied. Four individuals given 1 mc. P^{32} had

an average peak activity of 2.7 c.p.m. above background. There were no significant changes in the white blood counts of any of these patients during the time of study. Differential counts in all showed over 90 per cent lymphocytes. Figs. 7 and 8 depict the results obtained in 3 instances.

The results in a young woman with acute mixed monomyelocytic leukemia who was receiving 80 to 100 mg. of prednisone during the course of study are shown in Fig. 9. The very rapid and early delivery of labeled cells is apparent. Peak activity was 9.8 c.p.m. per γ-DNA-P.

Fig. 7.



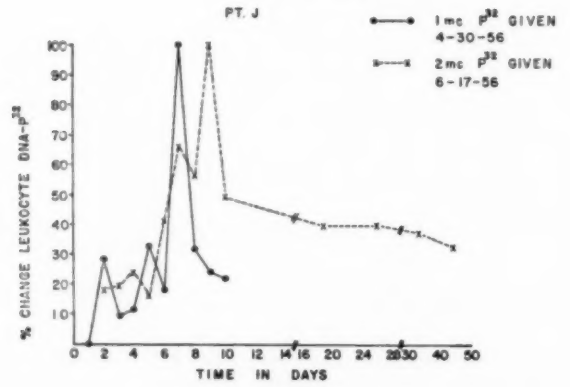


Fig. 8.

Fig. 7.—Irregularity of white cell P^{32} specific activity in a patient with chronic lymphocytic leukemia. Peak activity represents 2.75 c.p.m. per microgram DNA-P above background. Lymphocytes exceeded 95 per cent of the differential count.

Fig. 8.—A patient with chronic lymphocytic leukemia studied on two occasions. Peak activity for the 1 mc. dose was 3.67 c.p.m. and for the 2 mc. dose was 7.3 c.p.m.

Fig. 10 demonstrates the per cent change of leukocyte DNA-P^{32} activity in a patient with chronic myelocytic leukemia after treatment with 5 and 8 mc. of P^{32} on two different occasions. A therapeutic response was obtained each time. After the first dose the white count fell from 108,500 per c.mm. to 28,500 per c.mm. After the second dose it fell from 211,750 per c.mm. to

108,000 per c.mm. There were no significant changes in the differential count.

The rate of appearance of labeled cells was quite rapid on each occasion but the disappearance was relatively slow. In contrast to patients with chronic lymphocytic leukemia, DNA-labeling was quite high, reaching 43.5 and 103.9 c.p.m. per microgram DNA-P on the 5 and 8 mc. doses, respectively.

The results in this patient in an active phase of his disease are to be compared with those obtained in another individual (Patient Su.) with chronic myelocytic leukemia but requiring no therapy (Fig. 11). Note that the rate of appearance of labeled leukocytes in the latter is normal.

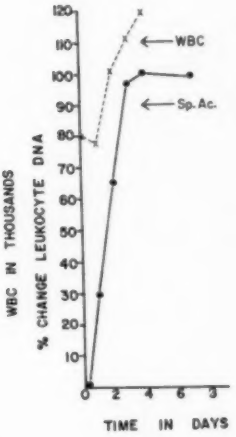


Fig. 9.

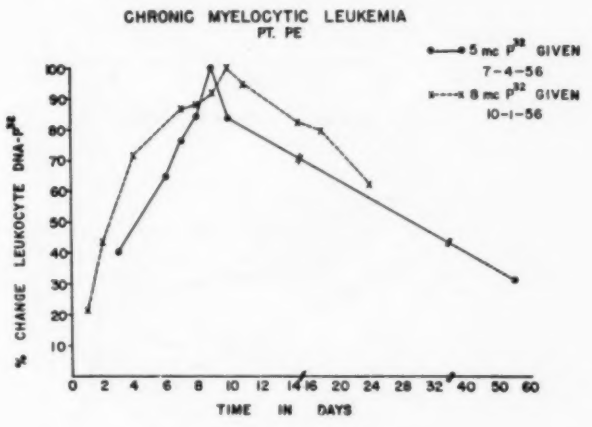


Fig. 10.

Fig. 9.—Rate of appearance of labeled white cells in a patient with acute monomyelocytic leukemia after administration of 500 μc P^{32}.

Fig. 10.—The rate of appearance and disappearance of P^{32}- labeled white cells in a patient with chronic myelocytic leukemia in relapse given therapeutic doses of P^{32} on two different occasions.

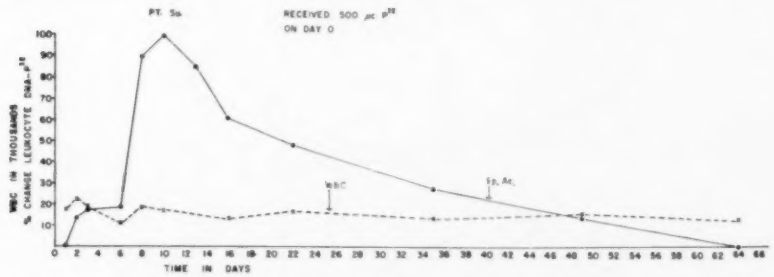


Fig. 11.—A patient with chronic myelocytic leukemia in remission demonstrating a curve for white cell DNA-P specific activity which appears quite normal.

The differences noted above in the rate of appearance of labeled leukocytes in the various diseases as compared to the normal are demonstrated in Fig. 12. The leukocyte DNA specific activity in patients with acute leukemia, chronic

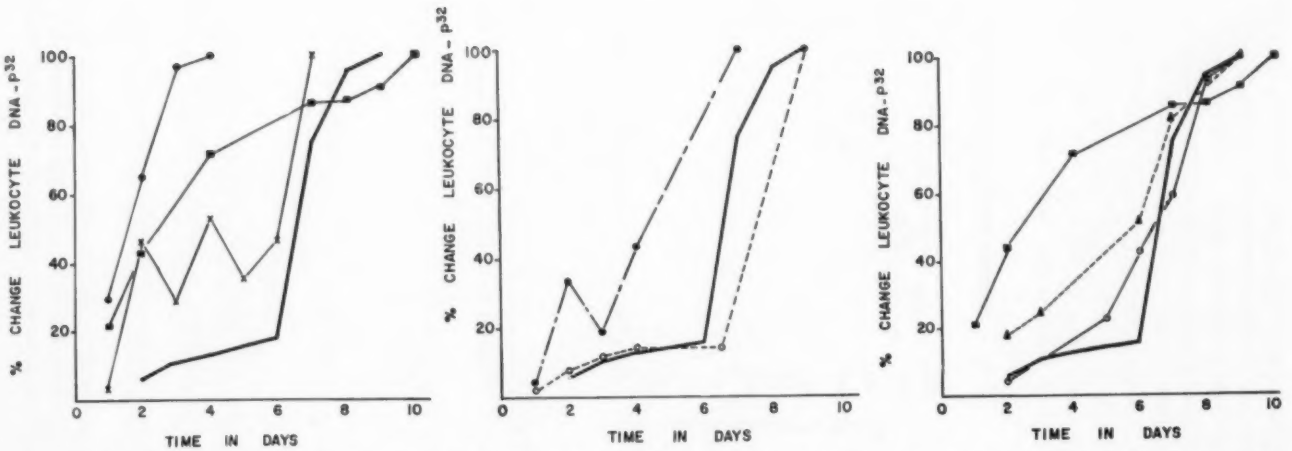


Fig. 12.—The rates of appearance of P^{32} - labeled white cells in a variety of conditions: —— normal; •——• acute leukemia; ■——■ chronic myelocytic leukemia (in relapse); x——x chronic lymphocytic leukemia; •- - • acute infection; ○——○ polycythemia vera; ○- - ○ chronic infection; ▲- -▲ myeloid metaplasia with leukemoid reaction.

myelocytic leukemia, chronic lymphocytic leukemia, and acute infection rose more rapidly than normal. The white cell entry rate in polycythemia vera and chronic infection was similar to that observed in the normal individual.

Fig. 13 shows the rates of disappearance of white cell radioactivity in various diseases. Aside from a slower decline in the curves for chronic myelocytic leukemia and myeloid metaplasia with a leukemoid reaction there is little difference among the various curves.

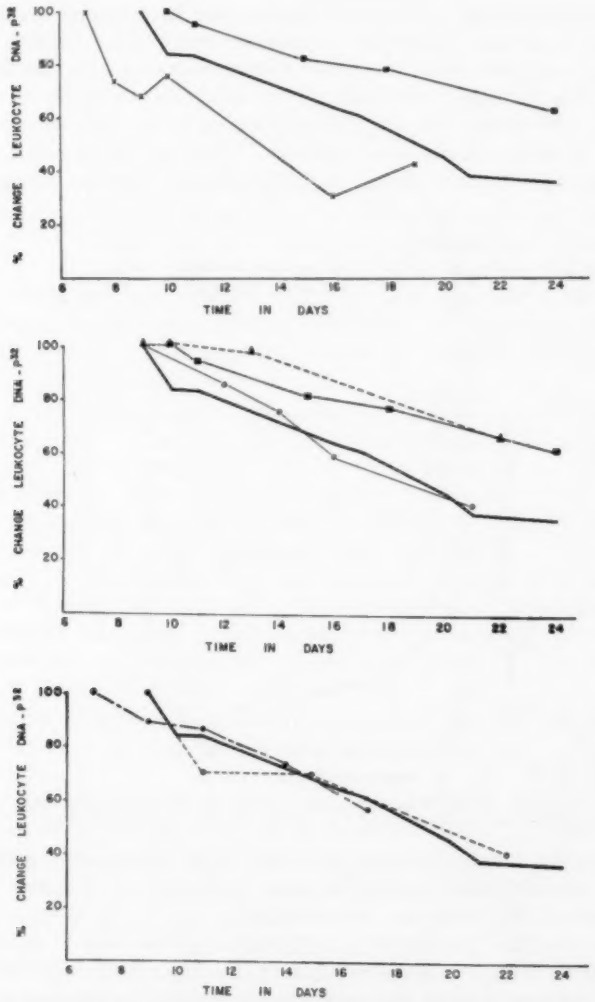


Fig. 13.—The disappearance rate of DNA-P^{32} labeled white cells in various diseases: —— normal; ■——■ chronic myelocytic leukemia (in relapse); x——x chronic lymphocytic leukemia; ▲- -▲ myeloid metaplasia with leukemoid reaction; ○——○ polycythemia vera; ● - - ● acute infection; ○ - - ○ chronic infection.

DISCUSSION

The lifespan of white blood cells may be conveniently divided into 3 periods: (1) the prevascular; (2) the intravascular; and (3) the postvascular period. With application of isotope methods of study, the first of these time intervals in the total lifespan of neutrophilic leukocytes, the prevascular phase, has been fairly well delineated. In this study, it has been shown that in the normal individual, the bulk of labeled leukocytes begin to make their appearance on the sixth day after administration of P^{32}, with a peak being reached on the eighth day. At this time, the number of labeled cells leaving the vascular tree apparently equals the number being delivered into the circulation. However, thereafter the rate of disappearance exceeds the rate of entry and there is a gradual "fall-off" of labeled cells. The mean age or blood lifespan of leukocytes may be determined from these data by the application of various mathematical formulas. This has been done by Kline and Cliffton,[1] Ottesen,[11, 15] and Osgood[6, 16, 17] as mentioned previously. Differences

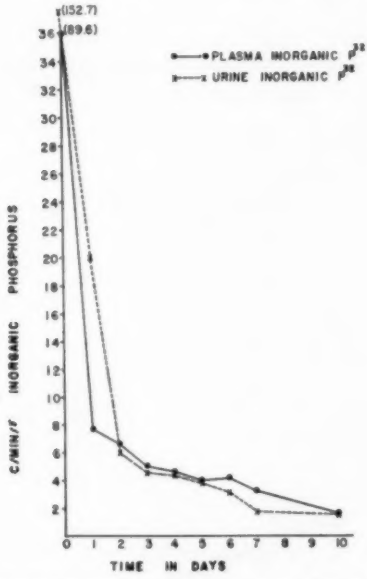


Fig. 14.—Clearance rate of P^{32} from the plasma of a normal individual after intravenous administration of 500 μc. The urine P^{32} falls concomitantly.

in results obtained by these investigators are explained largely by their interpretation of the effective tagging interval to which the dividing immature cells are exposed. Following the administration of a dose of P^{32}, the plasma is rapidly cleared of the isotope (Fig. 14). Up to 50 per cent is said to be excreted during the 6 days after injection and 1 to 2 per cent daily thereafter.[18] Whether the extremely brief high initial concentration or the long interval of low-level plasma P^{32}, or both, should be taken into consideration in lifespan

calculations is not known. If DNA uptake of P^{32} continues over a prolonged period then the decline in the leukocyte DNA-P^{32} represents a complex matter reflecting both disappearance and appearance of cells of varying specific activity. That cell re-entry may not be a significant factor in these considerations has been shown recently.[19]

There are still other considerations involved before any definite statements as to the lifespan of the white cell may be made. The leukocyte is subject to a great many influences, including changes in pH, electrolyte balance, hormones, and trapping in the capillary beds of the lungs[4] and at sites of infection and inflammation. Redistribution and local sequestration[20] following the administration of various substances,[19, 21-23] anaphylaxis, and stress are important. Finally, there is the tissue phase of leukocyte existence. All of these may alter the lifespan of the leukocyte and should be a consideration in calculating survival times.

In addition to the factors mentioned, certain limitations inherent in the use of isotope tracers for the study of "turnover" rates in general have been pointed out.[24, 25] Isotope uptake measurements may be misleading, especially since cells are in various stages of growth and degeneration and since nothing is known of the DNA precursors. Whether re-utilization of the P^{32} or of a DNA fraction occurs is not known, although there is some evidence to the contrary.[15] Another factor ignored in these studies is the radiation effect of P^{32} on cellular physiology.

Finally, the concept of an "appearance and disappearance" for P^{32}-labeled white cells may not be correct. As has been pointed out there is evidence to show that the intravascular lifespan of leukocytes may be quite short—a matter of minutes.[5] On this assumption, the leukocyte specific activity may merely be a reflection of the percentage of white cells labeled with P^{32} as they are delivered into the blood stream at any given moment. Hence, the degree of labeling in the blood would not necessarily be a measure of the intravascular lifespan of leukocytes. The peak specific activity on the eighth or ninth day may merely mean that the ratio of labeled to unlabeled cells appearing in the blood on that particular day is at its highest point. Thereafter, this ratio for entering leukocytes might appear to decline.

Realizing these as yet unsolved problems, it was felt that no statement as to the lifespan of the leukocyte is possible at present. Nevertheless, certain observations may be made from the data obtained. It is interesting to note the extremely rapid rate of appearance of labeled cells in patients with infection, acute leukemia, chronic myelocytic leukemia, and chronic lymphocytic leukemia as compared to the normal. White blood cells in leukemoid reactions appear to be intermediate in entry rate, while those in polycythemia rubra vera enter the vascular tree at a normal rate. Since those cells that appear early are not mature cells formed prior to P^{32} administration, their prevascular life must be quite brief in comparison to the normal. This is not difficult to understand when the cells found in the peripheral blood are immature morphologically as in the leukemias. Even in infection, release from

the bone marrow appears to occur not only at a greater rate but at an earlier period in the life of the cell. This is true even though a moderate number of band forms may be the only indication of an altered delivery into the circulation. Recent studies[14] with a nucleic acid stain have borne out the fact that, although morphologically identical, cells in the peripheral blood may differ in age and rate of release from the bone marrow.

The results obtained in patients with chronic lymphocytic leukemia are especially interesting in view of the extremely long lifespan postulated for lymphocytes in both normal individuals and those with chronic lymphocytic leukemia. The majority of lymphocytes in this disease are said to have a mean age of 100 to 200 days.[15, 16] This is surprising in view of the known numbers which enter the circulation through the thoracic duct. Yoffey[26] and Lawrence and his co-workers[27] demonstrated that the lymphocytes introduced via this route alone were able to replace those in the circulation approximately twice daily. Preliminary experiments in this laboratory show that DNA-P^{32}-labeled lymphocytes make their appearance in the thoracic duct of dogs as early as two hours after administration of the P^{32}. This indicates a remarkably rapid rate of maturation for this cell and makes it difficult to reconcile a long intravascular time interval for the lymphocyte with the known numbers of cells introduced. If labeled lymphocytes do appear rapidly in the blood after administration of the isotope, what is the explanation for the absence of significant leukocyte DNA-P^{32} activity in the first day or two? It may be that the labeled lymphocytes are so greatly diluted in the peripheral blood that the c.p.m. obtained is very little above background during that time. In lymphocytic leukemia, the delivery rate must be far greater, so that dilution is not as important a factor.

This type of isotope labeling may be used as a measure of the activity of the leukemic process in an individual, as suggested previously by Osgood and his co-workers.[16] Acute leukemias have a very rapid rate of delivery of white cells in contrast to controlled stable chronic leukemias. Chronic leukemias in relapse, however, appear to be intermediate in the time of appearance of new leukocytes.

SUMMARY

1. The technique of leukocyte DNA-P^{32} incorporation has been employed to study the rate of appearance and disappearance of white blood cells in normal individuals and in various diseases.

2. Leukocytes seem to enter the blood stream more rapidly than normal in acute leukemia, chronic myelocytic leukemia in relapse, chronic lymphocytic leukemia, acute infections, and in leukemoid reactions. The entry rate appears normal in chronic infections, polycythemia vera, and in chronic myelocytic leukemia in remission.

3. Problems important in any consideration of leukocyte lifespan determinations are discussed.

This work was performed with the technical assistance of Miss Gloria Paul, Miss Mary Baker, Mrs. Jeanine Green, and Mr. Robert Schrot.

REFERENCES

1. Kline, D. L., and Cliffton, E. E.: The Life Span of Leucocytes in the Human, Science **115**: 9, 1952.
2. Minot, G. R., and Isaacs, R.: Transfusion of Lymphocytes, J. A. M. A. **84**: 1713, 1925.
3. Lawrence, J. S., Ervin, D. M., and Wetrich, R. M.: Life Cycle of White Blood Cells, Am. J. Physiol. **144**: 284, 1945.
4. Weisberger, A. S., Heinle, R. W., Storaasli, J. K., and Hannah, R.: Transfusion of Leukocytes Labeled With Radioactive Phosphorus, J. Clin. Invest. **29**: 336, 1950.
5. White, L. P.: The Intravascular Life Span of Transfused Leukocytes Tagged With Atabrine, Blood **9**: 73, 1954.
6. Osgood, E. E., Li, J. G., Tivey, H., Duerst, M. L., and Seaman, A. J.: Growth of Human Leukemic Leukocytes In Vitro and In Vivo as Measured by Uptake of P^{32} in DNA, Science **114**: 95, 1951.
7. Lajtha, L. G., Oliver, R., and Ellis, F.: Incorporation of P^{32} and Adenine C^{14} into DNA by Human Bone Marrow Cells In Vitro, Brit. J. Cancer **8**: 367, 1954.
8. Hamilton, L.: Nucleic Acid Metabolism in Chronic Lymphatic Leukemia, J. Clin. Invest. **33**: 939, 1954.
9. Howard, A., and Pelc, S. R.: Nuclear Incorporation of P^{32} as Demonstrated by Autoradiographs, Exper. Cell Res. **2**: 178, 1951.
10. Taylor, J. H.: Autoradiographic Detection of Incorporation of P^{32} Into Chromosomes During Meiosis and Mitosis, Exper. Cell Res. **4**: 164, 1954.
11. Ottesen, J.: On the Age of Human White Cells in Peripheral Blood, Acta Physiol. Scandinav. **32**: 75, 1954.
12. Doering, P., Harbers, H., and Harbers, E.: Observations on Leukemic Blood Cell-DNA After In Vivo Labeling With P^{32}, Klin. Wchnschr. **35**: 31, 1957.
13. Craddock, C. G., Jr., Perry, S., Lawrence, J. S.: The Dynamics of Leukopoiesis and Leukocytosis as Studied by Leukopheresis and Isotopic Techniques, J. Clin. Invest. **35**: 285, 1956.
14. Perry, S., and Reynolds, J.: Methyl-Green-Pyronin as a Differential Nucleic Acid Stain for Peripheral Blood Smears, Blood **11**: 1132, 1956.
15. Christensen, B., and Ottesen, J.: The Age of Leukocytes in the Blood Stream of Patients With Chronic Lymphatic Leukemia, Acta Haemat. **13**: 289, 1955.
16. Osgood, E. E., Tivey, H., Davison, K. B., Seaman, A. J., and Li, J. G.: The Relative Rates of Formation of New Leukocytes in Patients With Acute and Chronic Leukemias, Cancer **5**: 331, 1952.
17. Osgood, E. E., and Krippaehne, M. L.: Comparison of the Life Span of Leukemic and Non-leukemic Neutrophils, Acta Haemat. **13**: 153, 1955.
18. Erf, L. A., Tuttle, L. W., and Lawrence, J. H.: Clinical Studies With the Aid of Radiophosphorus, Ann. Int. Med. **15**: 487, 1941.
19. Perry, S., Weinstein, I. M., Craddock, C. G., Jr., and Lawrence, J. S.: The Combined Use of Typhoid Vaccine and P^{32} Labeling to Assess Myelopoiesis, Blood **12**: 549, 1957.
20. Vejlens, G.: The Distribution of Leukocytes in the Vascular System, Acta path. et microbiol. scandinav. (Suppl.) **33**: 1, 1938.
21. Bierman, H. R., Kelly, K. H., Cordes, F. L., Byron, R. L., Polhemus, J. A., and Rapoport, S.: The Release of Leukocytes and Platelets From the Pulmonary Circulation by Epinephrine, Blood **7**: 683, 1952.
22. Bierman, H. R.: The Influence of Intra-arterial Administration of Histamine Upon the Circulating Leukocytes of Man, Blood **8**: 315, 1953.
23. Lawrence, J. S.: Leukopenia. A Discussion of Its Various Modes of Production, J. A. M. A. **116**: 478, 1941.
24. Siminovitch, L., and Graham, A. F.: Significance of Ribonucleic Acid and Deoxyribonucleic Acid Turnover Studies, Jour. Histo. and Cyto. **4**: 508, 1956.
25. Reiner, J. M.: The Study of Metabolic Turnover Rates by Means of Isotopic Tracers, Arch. Biochem. & Biophysics **46**: 53, 1953.
26. Yoffey, J. M.: Bone Marrow, Brit. Med. J. **2**: 193, 1954.
27. Adams, W. S., Saunders, R. H., and Lawrence, J. S.: Output of Lymphocytes in Cats Including Studies on Thoracic Duct Lymph and Peripheral Blood, Am. J. Physiol. **144**: 297, 1945.

EOSINOPHILIC RESPONSE TO INTRAPERITONEAL BLOOD

JOHN S. CHAPMAN, M.D., AND ROLLAND C. REYNOLDS, M.D.*
DALLAS, TEXAS

IN A PREVIOUS paper[1] it was shown that whole blood injected into the peritoneum of rats and mice regularly produced a reaction. One of the characteristics of this reaction was a striking eosinophil response which occurred regularly at 96 to 120 hours after injection of blood. This eosinophil response occurred regardless of clotting or nonclotting, and anticoagulants did not alter the response. The eosinophilic response occurred when the animal was injected with its own blood.

The present study was designed to determine what portion of whole blood was capable of producing the eosinophilic response and what portions were not.

METHODS

A group of white mice was each injected intraperitoneally with 1 ml. of human heparinized plasma. Six animals were sacrificed on the third, fifth, seventh, and ninth days. Smears were made from the peritoneum by drawing ordinary glass slides over the surface. These were stained with Wright's and a differential count was made in the usual way, never on less than 200 white blood cells, usually on 300 cells.

A similar group of white mice was injected with washed human red cells resuspended in saline. Groups of 6 were sacrificed at the same intervals and the peritoneal response was studied.

In order to test hemoglobin against stroma and to be certain that species difference was not a factor, rats were injected in the following manner. Approximately 2 ml. of whole rat blood was obtained by cardiac puncture and placed in a sterile heparinized tube. The sample was centrifuged at high speed for 5 minutes and the plasma removed. The remaining red cells were washed free of plasma by 3 washings of sterile physiologic saline. The volume was adjusted to the original sample volume with sterile saline, placed in the Deepfreeze, and subjected to alternate freezing and thawing for a period of 2 hours (30-minute intervals). After liberating the hemoglobin from the stroma, the sample was centrifuged at high speed for 10 minutes and the supernatant carefully removed with a Pasteur pipette. The supernatant was examined microscopically and found to be free of stroma. The hemoglobin of the supernatant was 7.0 to 9.5 Gm. per 100 ml. Of this material, 1.5 to 2.0 ml. was injected intraperitoneally into each of 10 mature white rats, each animal receiving hemoglobin prepared from its own blood. The animals were sacrificed at the end of 5 days and peritoneal smears prepared and counted in the manner described above.

From the William Buchanan Laboratory and the Departments of Internal Medicine and Pathology, The University of Texas Southwestern Medical School, Dallas, Texas.

This work was supported by grants-in-aid from the Buchanan Foundation and from the Dallas Tuberculosis Association.

Received for publication Aug. 26, 1957.

*Dr. Reynolds was the possessor of a U. S. Public Health Service Student Fellowship at the time this work was underway.

The same process as outlined above was repeated for the preparation of the stroma, with the exception that instead of adjusting to the original volume with saline, the volume was exceeded by the addition of a large volume of sterile distilled water. The red cells were allowed to lake in the distilled water for a period of 30 minutes and the sample centrifuged at high speed for a period of 5 minutes. The supernatant was carefully decanted and the process repeated until the residue gave a negative benzidine reaction. The remaining tan residue was suspended in saline to volume and injected into 6 white rats. Each animal received stroma prepared from its own blood. These animals were sacrificed at the end of 5 days and peritoneal smears made as before.

RESULTS

The comparative eosinophilic response of mice to human red blood cells as opposed to human plasma is illustrated graphically in Fig. 1. It will be noted that the peak of eosinophilic response occurs on the fifth day. At this point the t value for the difference lies between the 1 and 5 per cent level.

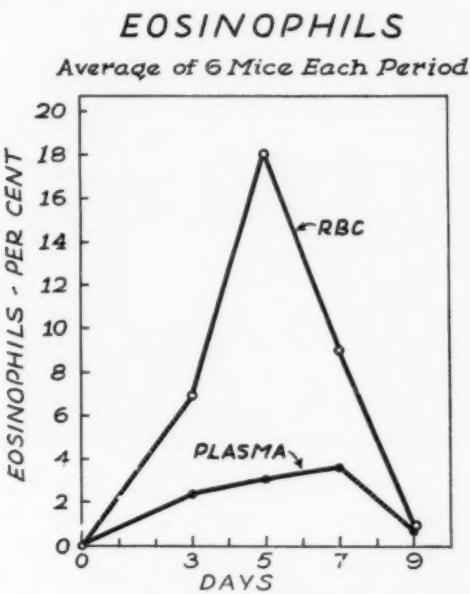


Fig. 1.—Comparative response of peritoneal reaction of mice to human plasma and to washed human red blood cells. The difference at 5 days is significant statistically.

The response of each rat to its own stroma and to its own hemoglobin is shown in Table I. Fig. 2 illustrates the striking difference in reaction. Smears from the peritoneum when hemoglobin was injected reveal very few cells, all of which are lymphocytes or mononuclears. Smears from peritoneum in contact with stroma present a much heavier exudate, rich in eosinophils, the nuclei of which exhibit unusual ring and horseshoe shapes, a point noticed by Bayne-Jones in regard to human fluids.[1-2] Granules, however, are typical.

The t value for the difference in reaction to autogenous hemoglobin and to autogenous stroma at the fifth day is significant at and beyond the 1 per cent level.

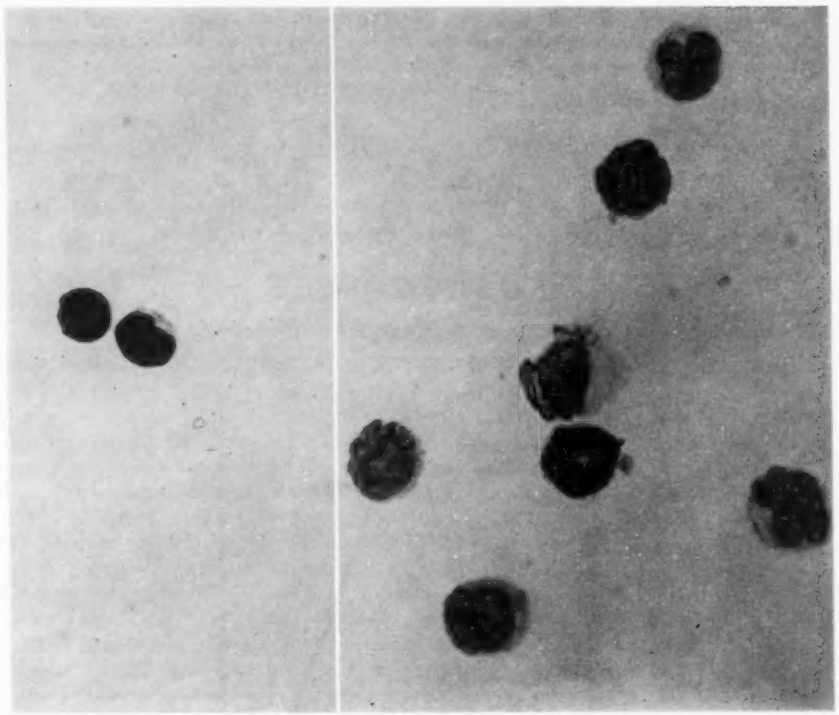


Fig. 2.—On the left is the very sparse lymphocytic response of the rat to its own hemoglobin. On the right the much more cellular response of the rat to its own red cell stroma.

TABLE I. PER CENT OF EOSINOPHILS IN PERITONEAL RESPONSE OF RATS TO AUTOGENOUS MATERIAL

	HEMOGLOBIN	R.B.C. STROMA	WHOLE BLOOD
	0.0	24.5	38
	0.0	13.0	20
	0.0	8.0	20
	2.0	9.0	34
	0.5	23.0	14
	0.5	10.5	
	0.5		
	0.0		
	0.0		
	0.0		
Number of animals	10	6	5
Mean	0.35 ± 0.63	14.8 ± 7.16	25.2 ± 10.27

DISCUSSION

It is apparent from the foregoing that whole blood injected intraperitoneally produces a characteristic eosinophilic response 5 days following injection. While plasma produces a weak response the major eosinophil-stimulating substance is associated with the red cell. It is also evident that hemoglobin plays no significant part in stimulation of eosinophils, but that substances attached to or composing the red cell stroma and membrane do evoke an eosinophilic response.

It cannot be said with certainty that all of the eosinophil-stimulating substance of whole blood is contained in the stromal portion. A mild eosinophilic response of the mouse peritoneum to such substances as commercial gamma globulin and bovine albumin has been demonstrated in this laboratory, and it is evident from Fig. 1 that plasma produces some degree of eosinophilic response. In all of these instances, however, it is possible that trauma associated with the injection may have led to some intraperitoneal bleeding, a source of error in the method which cannot be avoided. However, statistical comparison of the rat's response to its whole blood with its response to its stroma (Table I) indicates that there is no significant difference (t value 0.05 level).

The question of the role of hypersensitivity in the response to blood or its fractions is an obvious one. Pleural eosinophilia was produced by Weinberg and Seguin[3] and by Vaughn[4] by injection of various substances thought to be allergenic. But it has been noted[5, 6] that human pleural eosinophilia occurs under conditions in which the stimulus would seem to be the patient's own blood.

It would seem reasonable to suppose that if "allergy" or a similar process is involved blood from a different species should have a much more marked eosinophilic effect than the animal's autogenous blood. Statistical comparison of mouse reaction to human red cells with the rat's reaction to its own blood results in a t value of 1.17 where the value at the 5 per cent level is 2.262. It would thus seem that "allergy," as commonly understood, plays a minor role in the eosinophilic response of the peritoneum to blood and its components.

If this reasoning is correct it may be possible to postulate that red cell stroma contains a substance or substances which have specific eosinophil-stimulating capacity quite independent of "allergenic" properties. Work is now underway on fractions of stromal material to attempt further to isolate and identify this material.

CONCLUSIONS

1. It has been shown that the eosinophil-stimulating property of whole blood is chiefly, if not entirely, confined to the red cell.

2. It has been demonstrated that the eosinophil-stimulating property is associated with red cell membrane and stroma rather than with hemoglobin and other water soluble substances released by red cell lysis by freezing.

3. It is suggested that the eosinophil-stimulating substance may operate in other than strictly "allergic" fashion.

REFERENCES

1. Chapman, John S.: The Reaction of Serous Cavities to Blood, J. LAB. & CLIN. MED. **46**: 48-59, 1955.
2. Bayne-Jones, S.: Pleural Eosinophilia, With Report of a Case, Bull. Johns Hopkins Hosp. **27**: 12-16, 1916.
3. Weinberg, M., and Seguin, P.: Recherches biologiques sur l'éosinophilie, Ann. Inst. Pasteur **28**: 471-507, 1914.
4. Vaughn, J.: The Stimulation of the Eosinophil Leucocyte, J. Path. & Bact. **64**: 91-102, 1952.
5. Kabaker, I.: Contribution à l'étude expérimentale des pleurésies à éosinophiles au cours du pneumothorax artificiel, Strasbourg méd. **95**: 277, 300, 1935.
6. MacMurray, F. G., Katz, S., and Zimmerman, H. J.: Pleural Fluid Eosinophilia, New England J. Med. **243**: 330-334, 1950.

SERUM MACROGLOBULIN LEVELS IN RELATION TO AGE, SEX, AND DISEASE

NILS ERIKSEN, PH.D.
SEATTLE, WASH.

WHEN human serum is diluted several-fold with M/10 NaCl and analyzed ultracentrifugally, there is usually observed, in addition to the 4S and 7S components, a small amount of material with a sedimentation coefficient of about 19S. The latter component, termed macroglobulin or heavy component, has been shown by Wallenius and associates[1] to consist normally of two globulin fractions having electrophoretic maxima in the alpha-2 and gamma-1 regions, respectively. The alpha-2 portion is relatively sharply localized and is about twice the size of the other portion, which apparently extends slightly into both the beta and slower gamma regions. Individuals with extremely high concentrations of macroglobulin, presumably in this more diffuse fraction, were first reported by Waldenström,[2] who found the abnormality to be associated with a typical clinical syndrome which came to be known as macroglobulinemia. Since that time searches have been made for ultracentrifugal abnormalities in the serum protein patterns of various pathologic conditions. Perhaps the most extensive survey was that of Jahnke and Scholtan,[3, 4] who observed slight to moderate serum macroglobulin increases in nephrosis and certain liver diseases and decreases in multiple myeloma.

The present report is a summary of macroglobulin ($S > 15$) levels observed in the routine ultracentrifugal analysis of serum of healthy and diseased individuals from infancy to old age. As the study progressed a special effort was made to include patients known to have or suspected of having multiple myeloma. Another source of material was a group of asthmatic and other allergic individuals whose serum protein patterns were being studied electrophoretically.[10] Many of the patients were diagnostic problems whose sera were sent to this laboratory for electrophoretic and ultracentrifugal analysis. All cases with an obvious macroglobulinemia ($S > 15$) that was electrophoretically recognizable in the beta-gamma region have been excluded from consideration; these will be reported in a separate communication.

EXPERIMENTAL METHODS

Most of the serum specimens were obtained from individuals living or hospitalized in the Seattle area. Several specimens were sent from nearby cities by ordinary mail, and one

From the Department of Pathology, School of Medicine, University of Washington, Seattle, Wash.
Received for publication Sept. 6, 1957.

was sent by air mail from Chicago. All specimens were refrigerated at least overnight before analysis to reveal cryoglobulins that may have been present.

Electrophoresis.—Moving-boundary analyses were carried out at accurately determined serum dilutions (final dilution factor $\geqq 5$) in barbital buffer, ionic strength 0.1, pH 8.55 ± 0.05. Total protein values were calculated by use of a factor relating total electrophoretic area to the dry weight of solids retained by a dialyzing membrane. Details of the electrophoretic procedure and the determination of dry weights have been previously described.[5] The ratio between total solids (retained by a dialyzing membrane) and total electrophoretic area was found to be approximately constant, a coefficient of variation of 3.4 per cent having been determined for 10 specimens of whole or fractionated serum of different protein and lipoprotein content. All calculations were based on descending electrophoretic patterns.

When, in the later part of the study, electrophoretically separated fractions were desired for ultracentrifugal analyses, the center section of the cell was moved out of alignment at the end of the run. The whole assembly was removed from the water bath and dismantled down to the center section. Samples just large enough to fill the ultracentrifuge cell (about 1 ml.) were obtained with a needle and syringe after removal of unwanted overlaying liquid, as determined by inspection of the electrophoretic pattern. Owing to the probability of dilution and contamination resulting from turbulence accompanying the recovery of samples, only qualitative significance was attached to the ultracentrifugal analyses of these electrophoretically separated fractions.

Ultracentrifugation.—Analyses were done in the Spinco Model E ultracentrifuge at a speed setting of 59,780 r.p.m. and a bar angle of 60 degrees. Whole serum was diluted with 5 to 7 volumes of M/10 NaCl; serum fractions, other than those obtained electrophoretically, were ordinarily dialyzed against M/10 NaCl before analysis. Electrophoretically separated fractions were analyzed as obtained in barbital buffer. Runs were made without refrigeration or temperature control; the temperature was taken as the average value of rotor temperatures obtained at the beginning and end of each run. Experimentally observed sedimentation coefficients were adjusted only for variations in the viscosity of water, and were standardized at an apparent average rotor temperature of 25° C.

Approximate absolute concentrations were estimated from planimetric analyses of tracings of enlarged patterns, the total protein value being that obtained electrophoretically, as described above. Traces of flotation components occasionally observed were ignored. Both edges of the bar image were traced and average areas were computed for each component. Concentration variations arising from the sector shape of the cell were adjusted according to the square of the distance of the maximal ordinate from the center of rotation, but further corrections suggested by ultracentrifugal theory and practice were not made. The total protein concentrations at the serum dilutions used for analysis ranged from 0.8 to 1.6 Gm./100 ml., with the exception of 9 specimens, mostly from cases of multiple myeloma, in which the concentrations ranged from 1.8 to 2.2 Gm./100 ml. In most instances the macroglobulin content of the diluted serum was less than 0.1 Gm./100 ml., and in a few instances it was near or below the limit of detection. An uncertainty of about ± 0.05 Gm./100 ml. was estimated for calculated values of macroglobulin concentrations in undiluted serum. The error of measurement being relatively large at these low concentrations, individual values must be regarded only as approximations of the true macroglobulin levels. Moreover, the measured areas may have represented not a single ultracentrifugal component but an unresolved group of components with a small range of sedimentation coefficients.

A few preparative flotation or sedimentation runs were made at 30,000 r.p.m. in the Spinco Model L ultracentrifuge equipped with the 30.2 rotor, under refrigeration. The flotation technique was essentially that of Lindgren, Elliott, and Gofman.[6] The sedimentation concentrates were prepared from suitable dilutions of serum in M/10 NaCl. The duration of the run and the volume of top layer removed were empirically chosen. In some instances the bottom layer was diluted with salt solution and recycled to achieve a greater relative concentration of heavier particles.

RESULTS AND DISCUSSION

Macroglobulin levels in 151 serum specimens were determined; the results are summarized in Tables I, II, and III. The 4 highest values ($\geqq$ 1.0 Gm./100 ml.) were found in patients with the nephrotic syndrome. All extremely low

TABLE I. SERUM MACROGLOBULIN LEVELS OF MEN

GROUP	NO. CASES	AGE (YR.)		MACROGLOBULIN (GM./100 ML.)		
		RANGE	MEAN	RANGE	MEAN	S. D.
Normal	4	25-41	35	0.23-0.28	0.26	0.024
Asthma, hay fever, urticaria	10	24-68	38	0.16-0.36	0.24	0.052
Miscellaneous*	26	19-88	62	0.14-0.46	0.24	0.070
Multiple myeloma	17	50-85	68	0.00-0.21	0.15	0.054
Single cases						
Disseminated lupus erythematosus with nephrotic syndrome			24		0.49	
Diabetes mellitus with chronic lymphatic leukemia and arteriosclerotic disease			64		0.63	

*Each case had at least one of the following features: chronic unexplained anemia, acquired hemolytic anemia, acute plasma-cell leukemia, chronic lymphatic leukemia, pancytopenia, hilar adenopathy, Hodgkin's lymphoma, Paget's disease, atypical marrow plasmacytosis with α_2-hyperglobulinemia, disseminated lupus erythematosus, rheumatoid arthritis, purpura with cryoglobulinemia, cerebrovascular accident, arteriosclerotic disease, familial hypercholesterolemia with xanthoma tendinosum, chronic unexplained lung disease, multiple kidney infarcts with renal blood-loss, basal-cell carcinoma of lip, prostatic carcinoma, localized bone lesions with 10S macroglobulinemia, fractured femur, hemorrhoids, syphilis.

values and the majority below 0.2 Gm./100 ml. were found in patients with multiple myeloma. These findings are consistent with the experience of Waldenström,[7] as well as that of Jahnke and Scholtan.[4] Apparently significant elevations of macroglobulin levels were observed in single cases of nephrogenic diabetes insipidus (child), Niemann-Pick (?) disease (child), multiple myeloma accompanied by the nephrotic syndrome (woman), and diabetes mellitus accompanied by chronic lymphatic leukemia and arteriosclerotic disease (man); all 4 values were in the range 0.60 to 0.65 Gm./100 ml. Waldenström[7] has observed increases in some cases of disseminated lupus erythematosus, but none of the 4 cases of this disease reported here had obviously elevated values; the highest was 0.49 Gm./100 ml., found in a man who also had the nephrotic syndrome. Since only 2 cases of recognized liver disease were encountered, previously reported increases[4] could not be verified; in a woman with multiple myeloma and portal cirrhosis the observed value was 0.41 Gm./100 ml., which was the highest value found among the myeloma cases with the exception of the patient who also had the nephrotic syndrome. Cryoglobulin was noted in 7 specimens, in only two of which was the amount massive and both were from cases of myeloma; in none of these cases was evidence obtained to indicate that the cryoglobulin was mainly, if at all, in the macroglobulin fraction. In 13 specimens traces or small amounts of material in the 8-14S class were observed, and in 2 specimens a major peak was found in this class. Sixteen specimens outside the myeloma group were characterized by hypergammaglobulinemia ($>$ 2 Gm./100 ml.), but there was no obvious correlation between the macroglobulin and γ-globulin levels. More specifically, Wallenius and associates[8] found that the amount of gamma-1 macroglobulin in pathologic sera was

not correlated with γ-globulin increases; they did observe, however, at least a tenfold reduction of the gamma-1 macroglobulin in the sera of 2 patients with agammaglobulinemia. In the present series the lowest serum macroglobulin value among all infants and children was found in a boy with extreme hypogammaglobulinemia.

TABLE II. SERUM MACROGLOBULIN LEVELS OF WOMEN

GROUP	NO. CASES	AGE (YR.) RANGE	AGE (YR.) MEAN	MACROGLOBULIN (GM./100 ML.) RANGE	MACROGLOBULIN (GM./100 ML.) MEAN	MACROGLOBULIN (GM./100 ML.) S. D.
Normal	5	21-49	35	0.30-0.36	0.33	0.026
Asthma, hay fever, urticaria, furunculosis, eczema	19	16-65	39	0.26-0.46	0.34	0.056
Miscellaneous*	14	21-92	57	0.17-0.48	0.31	0.099
Multiple myeloma	12	43-85	66	0.00-0.27	0.16	0.080
Single cases						
Multiple myeloma with nephrotic syndrome			51		0.65	
Chronic renal disease with nephrotic syndrome			62		1.04	
Multiple myeloma with portal cirrhosis			71		0.41	

*Each case had at least one of the following features: chronic unexplained anemia, reticuloendothelial hyperplasia with plasma-cell infiltration, splenomegaly, pancytopenia, disseminated lupus erythematosus, rheumatoid arthritis, purpura with cryoglobulinemia, severe petechiae with 10S macroglobulinemia, Felty's syndrome, recurrent hematomata, biliary cirrhosis, familial hypercholesterolemia with xanthelasma, Sjögren's syndrome, acute parotitis.

TABLE III. SERUM MACROGLOBULIN LEVELS OF INFANTS AND CHILDREN

GROUP	NO. CASES	AGE (YR.) RANGE	AGE (YR.) MEAN	MACROGLOBULIN (GM./100 ML.) RANGE	MACROGLOBULIN (GM./100 ML.) MEAN	MACROGLOBULIN (GM./100 ML.) S. D.
Normal (cord serum)	8		0	0.35-0.49	0.39	0.047
Normal	11	0.5-12	7	0.36-0.52	0.43	0.052
Chronic asthma	5	2-8	6	0.39-0.49	0.44	0.036
Recurrent infections (upper respiratory, middle-ear, skin, urinary tract)	7	2-11	6	0.34-0.48	0.42	0.049
Nephrotic syndrome	3	1.5-11	6	1.00-1.21	1.10	0.105
Single cases						
Letterer-Siwe's disease			0.5		0.36	
Nephrogenic diabetes insipidus			0.5		0.60	
Recurrent infections with extreme hypogammaglobulinemia			6		0.31	
Cellulitis following mastoiditis			8		0.34	
Niemann-Pick's (?) disease with hypergammaglobulinemia			9		0.65	

Three serum specimens were subjected to ultracentrifugal flotation separations at a solvent density of 1.06. Two of the specimens were from cases of familial hypercholesterolemia and the third was from the case of multiple myeloma with the nephrotic syndrome. The apparent recoveries of macroglobulin in the ultracentrifugal bottom fractions ranged from 75 to 100 per cent, indicating that, in these cases at least, the macroglobulin was not predominantly a low-density lipoprotein.

Statistical "t" tests applied to the grouped data of Tables I and II showed that, with the exception of the myeloma group, the mean serum macroglobulin level of the women was greater than that of the men ($P < 0.01$, except in the miscellaneous group, where $P < 0.02$), age and disease biases being recognized as possibly existent but assumed negligible. In both sexes the mean level of the myeloma group was less than that of any of the other groups ($P < 0.01$).

Fig. 1.—Ultracentrifugal patterns of serum diluted sevenfold, after 8 minutes at top speed: normal male child (upper), normal male adult (middle), and male adult with multiple myeloma showing no detectable macroglobulin (lower).

With the exception of 5 obviously elevated values, all serum macroglobulin levels of infants and children were in the range 0.31 to 0.52 Gm./100 ml. No significant differences were found between sexes or between the groups listed in Table III, the nephrotic group excepted. Normal infants and children had higher values than normal women, and children with asthma or recurrent infections had higher values than women with roughly comparable conditions ($P < 0.01$). The difference between the newborn and normal women was less significant ($0.02 < P < 0.05$). Differences between children and male adults would obviously be more highly significant.

Ultracentrifugal patterns of serum from a normal child, normal man, and myeloma patient with no detectable macroglobulin are compared in Fig. 1 (macroglobulin denoted by M in illustrations).

Electrophoretic separation of the alpha-2 and gamma macroglobulins was attempted in 35 cases, including 4 normal children, 15 myeloma patients, and 16 miscellaneous patients (nephrosis not represented). In all cases the sample obtained from the ascending side just above the γ-globulin boundary (α_2-globulin sample) contained macroglobulin, and in 33 cases the amount was definitely greater than that found in the corresponding sample obtained from the descending side just above the α_2-globulin boundary (γ-globulin sample). If a

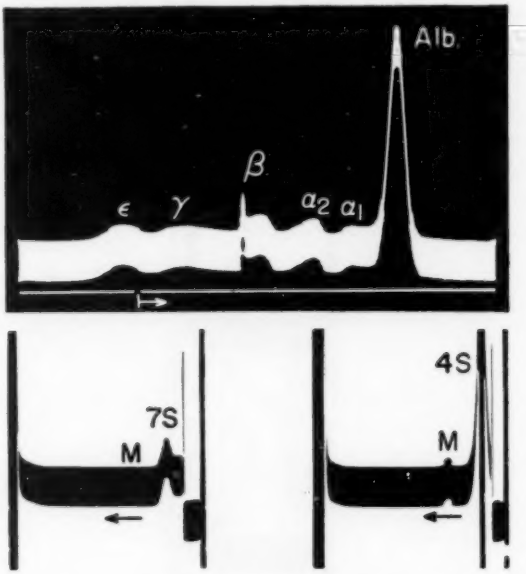


Fig. 2.—Descending electrophoretic pattern of serum from normal male child (upper), and ultracentrifugal patterns of electrophoretically separated descending (left) and ascending (right) fractions, after 10 minutes at top speed. See text for details.

specimen happened to contain a significant amount of beta macroglobulin it probably would be present to some extent in both samples. In 11 cases, of which 9 were multiple myeloma, no macroglobulin was detected in the γ-globulin sample. In one case the macroglobulin was about equally distributed between the 2 samples (child with Niemann-Pick [?] disease), and in another slightly more macroglobulin was found in the γ-globulin than in the α_2-globulin sample (man with Hodgkin's lymphoma; total macroglobulin = 0.46 Gm./100 ml.). In both of these latter cases the serum contained γ-globulin in excess of 2 Gm./100 ml. The distribution of macroglobulin, as revealed by this sampling procedure, in a normal child's serum is illustrated in Fig. 2. The electrophoretic appearance of the α_2-globulin component is generally characteristic of the serum protein patterns of infants and children as obtained in this laboratory; the concentration of material in the leading portion of the asymmetric peak may correspond to the relatively high concentration of macroglobulin

ordinarily observed ultracentrifugally. Bernfeld and Stefanini[9] observed α_2-globulin peaks of similar shape in serum from cases of hemophilia and idiopathic thrombocytopenic purpura.

The localization of macroglobulin in the serum protein pattern of a nephrotic child, as determined by differential preparative ultracentrifugation, is illustrated in Fig. 3. In this case the macroglobulin appeared to constitute a major part of the (slow) α_2-globulin component, the dilutions of the electrophoretic samples having been made equivalent.

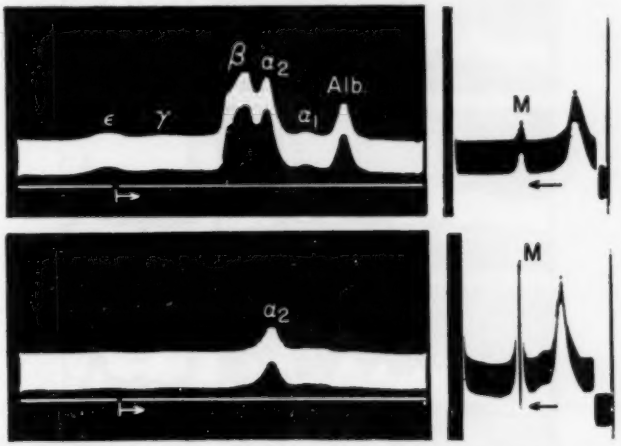


Fig. 3.—Electrophoretic and ultracentrifugal patterns of serum (upper) and macroglobulin concentrate (lower) from a child with the nephrotic syndrome. The ultracentrifugal photographs were taken after 22 and 31 minutes at top speed, respectively. The macroglobulin concentrate was analyzed directly in the ultracentrifuge, and diluted and dialyzed for electrophoresis (lower left).

A serum specimen possibly having a double macroglobulin component in the alpha-2 region is illustrated by electrophoretic and ultracentrifugal patterns in Fig. 4. The serum was from the male adult with diabetes mellitus accompanied by chronic lymphatic leukemia and arteriosclerotic disease. The whole serum had an obviously increased α_2-globulin complex, partially resolved into 2 components, the slower of which was the larger. The preparative ultracentrifugal bottom fraction, containing practically all of the macroglobulin, had a relatively concentrated α_2-globulin component with a flat top (a slight resolution was evident in the ascending pattern, not shown), possibly representing 2 components of approximately equal concentrations and slightly different mobilities. The preparative ultracentrifugal top fraction, containing only a trace of macroglobulin, had an α_2-globulin component diminished in both portions, the slower again having been the larger.

The difference between serum macroglobulin levels of men and women, although apparently significant, is generally too small to be consistently revealed by comparison of routine electrophoretic patterns. Both parts of the macroglobulin complex migrate electrophoretically in company with several

ultracentrifugally slower components, which may vary independently and, together, may easily obscure the macroglobulin. From a quantitative point of view the bulk of the difference is more likely to occur in the α_2-globulin fraction, where the major part of the macroglobulin is usually found, than in the γ-globulin fraction.

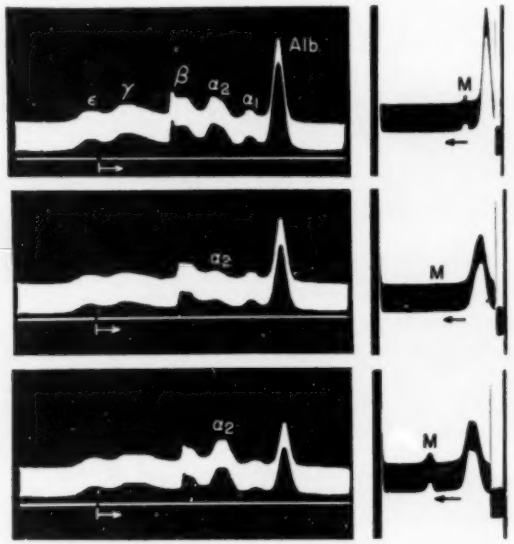


Fig. 4.—Electrophoretic and ultracentrifugal patterns of serum (upper), and preparative ultracentrifugal top (middle) and bottom (lower) fractions, showing concentration of apparently complex macroglobulin in the alpha-2 region. The ultracentrifugal photographs were taken after 8, 20, and 20 minutes at top speed, respectively. See text for details.

In multiple myeloma the γ-macroglobulin level appears to be consistently reduced, but reductions in the α_2-macroglobulin level are not infrequent in this disease; in several cases only a trace was found in the electrophoretically separated α_2-fraction, and in 2 cases the total macroglobulin was below the limit of detection. Ten miscellaneous cases (9 men) in which multiple myeloma was unconfirmed or unsuspected had serum macroglobulin levels less than 0.2 Gm./100 ml. It may be that multiple myeloma is only one of a number of diseases in which the macroglobulin level is commonly depressed.

Insofar as elevations of serum macroglobulin levels are concerned, the most frequent finding is an association of high α_2-macroglobulin levels with nephrosis. In view of the probable heterogeneity of this fraction it seems likely that elevations will be consistently found in various other conditions also. Sizable increases of macroglobulin in the beta and gamma regions are conveniently embraced by the ever broadening entity termed macroglobulinemia, having massive elevations of serum macroglobulin at its core. Whether an α_2-macroglobulinemia ever occurs as a specific characteristic of Waldenström's syndrome is a question that remains to be answered.

SUMMARY

Macroglobulin ($S > 15$) levels were determined in 151 serum specimens; patients with Waldenström's macroglobulinemia or apparently related conditions have been excluded from the study.

The mean value for healthy and many diseased adults was in the vicinity of 0.3 Gm./100 ml. Women generally had a slightly higher level than men. The mean value for infants and children, obvious pathologic elevations excluded, was approximately 0.4 Gm./100 ml., which was found to be significantly higher than the mean adult value.

High values were consistently found in patients with the nephrotic syndrome, and low values were consistently found in patients with multiple myeloma. High or low values were observed in various other conditions, but additional trends, if present, could not be verified owing to the heterogeneity of the case material and the frequent lack of definitive diagnoses.

The electrophoretic distribution of the macroglobulin was investigated, and both the α_2-globulin and γ-globulin fractions were implicated as sites of significant variations in the macroglobulin level of the cases studied.

I am grateful to numerous physicians in the Seattle area and elsewhere for making specimens and case histories available to me, and especially to Drs. Arno G. Motulsky and Wade Volwiler for their active interest in this work.

REFERENCES

1. Wallenius, G., Trautman, R., Kunkel, H. G., and Franklin, E. C.: Ultracentrifugal Studies of Major Non-lipide Electrophoretic Components of Normal Human Serum, J. Biol. Chem. **225**: 253-267, 1957.
2. Waldenström, J.: Incipient Myelomatosis or "Essential" Hyperglobulinemia With Fibrinogenopenia—a New Syndrome? Acta med. scandinav. **117**: 216-247, 1944.
3. Scholtan, W.: Klinische Ultrazentrifugen-Studien. I. Mitteilung. Ultrazentrifugendiagramm des normalen menschlichen Serums, Ztschr. d. ges. exper. Med. **121**: 574-588, 1953.
4. Jahnke, K., and Scholtan, W.: Klinische Ultrazentrifugen-Studien. II. Mitteilung. Pathologische Veränderungen im Serum-Ultrazentrifugen-Diagramm, Ztschr. d. ges. exper. Med. **122**: 39-59, 1953.
5. Eriksen, N., Ellerbrook, L. D., and Lippincott, S. W.: Serum Protein Analysis by Electrophoresis and by the Wolfson-Cohn Chemical Method, Clin. Chem. **2**: 334-346, 1956.
6. Lindgren, F. T., Elliott, H. A., and Gofman, J. W.: The Ultracentrifugal Characterization and Isolation of Human Blood Lipids and Lipoproteins, With Applications to the Study of Atherosclerosis, J. Phys. & Coll. Chem. **55**: 80-93, 1951.
7. Waldenström, J.: Abnormal Proteins in Myeloma, Ad. Int. Med. **5**: 398-440, 1952.
8. Wallenius, G., Trautman, R., Franklin, E. C., and Kunkel, H. G.: Heavy Components of Human Serum, Fed. Proc. **15**: 378, 1956.
9. Bernfeld, P., and Stefanini, M.: Characteristic Electrophoretic Patterns in Hemophilia and Idiopathic Thrombocytopenia, Proc. Soc. Exper. Biol. & Med. **77**: 551-553, 1951.
10. Stroh, J. E., and Eriksen, N.: Serum Electrophoresis Patterns in the Hypersensitive State, Ann. Allergy. (In press.)

THE EFFECT OF CORTICOTROPHIN, CORTISONE, AND PREDNISONE ON WHOLE BLOOD COAGULATION TIME

F. Dreyfuss, M.D., and J. Menczel, M.D.
Jerusalem, Israel

CORTICOTROPHIN and cortisone have been widely suspected as a cause of thromboembolic disorders. The statistical evaluation of this relationship is not easy since comparable series of cases are usually unavailable; most of the diseases treated with these preparations run a prolonged and frequently severe course and may by themselves be predisposing to thromboembolic complications. Thus the occasional observation of thromboembolic phenomena in a group of patients treated by these hormones, sometimes in combination with antibioties, does not necessarily prove the thrombogenic nature of these substances.

The literature dealing with the action of corticotrophin and cortisone upon blood coagulation by no means presents a clear picture. Smith and his co-workers[1] as well as de Takats and Voigt[2] found variable changes in clotting time after the administration of corticotrophin. Cosgriff and his co-workers[3] described accelerated clotting, an observation confirmed by others.[4, 5] Similarly, Bounameaux and his group[6] noted that cortisone induced quicker clotting, but found that corticotrophin only rarely elicited such a reaction. Increased activity of factor VII (proconvertin) after corticotrophin and cortisone was noted by de Nicola.[7] Others,[8-10] however, could not discover any significant alteration of clotting time after the administration of these hormones. An antagonistic effect of cortisone and corticotrophin on the anticoagulant action of ethyl biscoumacetate has been reported:[11, 12]

Increase of the thrombocyte count has been observed following hormone administration,[13] including thrombocytopenic leukemic patients.[14, 15] Others have seen thrombocytopenia[16] or no change in platelet counts[14] after the injection of corticotrophin.

In view of this conflicting evidence, and because of the possible relationship of thromboembolic disease to stress in general[18] and to a state of hypercoagulability, further studies of the influence of these "stress mediating" hormones on coagulation may be of interest. However, even if proved hypercoagulability represents only one aspect, although an important one (Virchow), of the multifaceted picture of thrombosis formation.

A previous study[19] of the influence of emotional stress on the coagulation time in healthy individuals had clearly demonstrated the superiority of a method

From the Department of Medicine "A" of the Rothschild Hadassah University Hospital and the Hebrew University Hadassah Medical School, Jerusalem, Israel.

This investigation was supported by a grant from the Hadassah Medical Organization research funds.

Received for publication April 9, 1957.

using siliconized test tubes over the glass tube method, as in common use. For this reason, both methods were used in contrast to most previously published studies. The fibrinogen B test of Cummine and Lyons[20] was also employed because of its simplicity and because of the claim of the authors—disputed by others—that it is a means of detecting a prethrombotic state, whenever a positive test is accompanied by a shortened coagulation time.

MATERIALS AND METHODS

Short-term experiments performed on ward patients with various illnesses who had not recently received any of the hormones under investigation are reported. The patients ate a light breakfast before the experiment; care was taken to avoid hunger by giving tea and rusks whenever requested. Prior to and 4 hours after the intramuscular administration of corticotrophin, cortisone, or saline, absolute eosinophil and thrombocyte counts were taken. At the same times, as well as 2 hours after the injection, the whole blood coagulation time was tested, in glass and in siliconized tubes, and the beta naphthol test was performed. The effect of prednisone given orally was studied in the same fashion. No report of the influence of this material on the clotting time has come to our attention. The effect of these substances as well as of a saline injection was observed in the same 22 individuals. It was unavoidable that the technician was frequently aware of the substance tested.

Clotting Time Determination.—Venous blood was obtained by a smooth puncture (needle bore 20). About 12 ml. was withdrawn and the coagulation time was determined in 2 glass and 2 siliconized tubes (size 10 × 100 ml.) in a water bath at 37° C., each one containing 1 ml. of blood. Syringes and needles were siliconized* and used only once more after resterilization. The zero point taken was that moment when half of the blood had entered the syringe. The glass tubes were tilted every half minute, the siliconized tubes every minute; observation of clotting in the siliconized tubes was started only after 5 minutes had elapsed. The second glass and siliconized tube were only tilted after the first ones had shown clotting. Care was taken not to use the first few milliliters of blood entering the syringe.

Fibrinogen B Test.—The method followed was that of Cummine and Lyons.[20] Five drops of a 2 per cent beta naphthol solution in 50 per cent alcohol was added to 1 ml. of oxalated plasma and after 5 and 10 minutes the amount of precipitate, if any, was noted. Normal plasma does not show any precipitate within such a time period. The result was then graded as negative, one plus, or two plus.

Cell Counts.—Eosinophil counts were done using a 0.01 per cent eosin and 5 per cent acetone stain in distilled water, thrombocyte counts with 1 per cent sequestrene (disodium-ethylenediaminetetraacetic acid) in 0.8 per cent sodium chloride solution.

RESULTS

The results of the tests performed with the 3 subtances under investigation and of the controls are summarized in Tables I and II. Table III lists the individual results of the tests performed with prednisone, because we found it to be the most potent of the materials examined and since no previous studies with this compound have been reported.

For the evaluation of the change of coagulation time, either the 2- or the 4-hour value has been used, employing whichever would show the more pronounced trend in comparison with the initial determination. The reason for this seemingly arbitrary selection is the fact that it is by no means certain whether the 4-hour point, as, e.g., used in the Thorn test, would necessarily be the one to exhibit maximal prolongation or shortening of the time in each instance. In

*Silicone resin, Drifilm 9986, General Electric Corporation.

TABLE I. SUMMARY OF RESULTS OF SILICONE COAGULATION TIME (S.C.T.) DETERMINATIONS AND ABSOLUTE EOSINOPHIL COUNTS AFTER CORTICOTROPHIN, CORTISONE, PREDNISONE, AND NORMAL SALINE SOLUTION

SUBSTANCE	DOSE	NO. OF CASES	DECREASE OF S.C.T.			NO CHANGE OF S.C.T.		INCREASE OF S.C.T.			EOSINOPHILS (NO. OF CASES)		
			NO. OF CASES	(PER CENT)	AVERAGE DECREASE IN MIN.	NO. OF CASES	(PER CENT)	NO. OF CASES	(PER CENT)	AVERAGE INCREASE IN MIN.	DE-CREASE	NO CHANGE	IN-CREASE
Corticotrophin (intramuscular)	25 mg.	10	6	60.00	5.00	0	0.00	4	40.00	6.75	6	2	2
	50 mg.	30	24	80.00	5.16	1	3.30	5	16.66	5.60	26	4	0
Cortisone (intramuscular)	50 mg.	27	21	77.70	4.90	0	0.00	6	22.20	5.00	14	5	8
	100 mg.	2	2	100.00	5.00	0	0.00	0	0.00	0.00	0	0	2
Prednisone (oral)	5 mg.	1	1	100.00	4.00	0	0.00	0	0.00	0.00	1	0	0
	10 mg.	30	25	83.33	5.52	3	10.00	2	6.60	4.00	24	4	2
	15 mg.	2	2	100.00	8.00	0	0.00	0	0.00	0.00	2	0	0
	20 mg.	1	1	100.00	3.00	0	0.00	0	0.00	0.00	1	0	0
Controls normal saline solution (intramuscular)	2 ml.	48	20	41.66	2.20	10	20.83	18	37.5	2.77	7	29	12

TABLE II. SUMMARY OF RESULTS OF GLASS COAGULATION TIME (G.C.T.), DETERMINATIONS AND ABSOLUTE EOSINOPHIL COUNTS AFTER CORTICOTROPHIN, CORTISONE, PREDNISONE, AND NORMAL SALINE SOLUTION

SUBSTANCE	DOSE	NO. OF CASES	DECREASE OF G.C.T.			NO CHANGE OF G.C.T.		INCREASE OF G.C.T.			EOSINOPHILS (NO. OF CASES)		
			NO. OF CASES	(PER CENT)	AVERAGE DECREASE IN MIN.	NO. OF CASES	(PER CENT)	NO. OF CASES	(PER CENT)	AVERAGE INCREASE IN MIN.	DECREASE	NO CHANGE	INCREASE
Corticotrophin (intramuscular)	25 mg.	10	5	50.00	2.80	0	0.00	5	50.00	1.20	6	2	2
	50 mg.	15	8	53.33	2.13	3	20.00	4	26.66	0.82	15	0	0
Cortisone (intramuscular)	50 mg.	6	4	66.66	1.82	1	16.66	1	16.66	1.00	3	0	3
Prednisone (oral)	5 mg.	1	1	100.00	3.00	0	0.00	0	0.00	0.00	1	0	0
	10 mg.	25	17	68.00	1.84	2	8.00	6	24.00	1.33	21	2	2
	15 mg.	2	1	50.00	1.30	0	0.00	1	50.00	1.00	2	0	0
	20 mg.	1	0	0.00	0.00	0	0.00	1	100.00	1.00	1	0	0
Controls normal saline solution (intramuscular)	2 ml.	16	8	50.00	0.91	4	25.00	4	25.00	1.07	4	9	3

TABLE III. RESULTS OF GLASS (G.C.T.) AND SILICONE (S.C.T.) COAGULATION DETERMINATIONS AND EOSINOPHIL AND THROMBOCYTE COUNTS BEFORE AND AFTER ORAL ADMINISTRATION OF PREDNISONE

NO.	PATIENT	SEX	DIAGNOSIS	G.C.T. IN MINUTES			S.C.T. IN MINUTES			TOT. EOS. COUNT IN MM.^{3}		THROMBOCYTE COUNT IN MM.^{3}	
				BEFORE	2 HR. AFTER	4 HR. AFTER	BEFORE	2 HR. AFTER	4 HR. AFTER	BEFORE	4 HR. AFTER	BEFORE	4 HR. AFTER
							10 mg. Prednisone						
1	Sh. W.	F.	Thrombocytopenic purpura	6-8^{30}	5^{30}-7^{30}	5^{30}-7^{30}	26-30	8-18	9-11	396	88	66.000	62.000
2	G. Ch.	F.	Hiatus hernia	5^{30}-7	4^{30}-5^{30}	5^{30}-6	12-15	5-6	9-12	132	176	115.000	137.000
3	K. T.	F.	Postcholecystectomy, pyelonephritis, diab. mel.	5^{30}-9	5-6	5-8	15-19	11-13	8-18	88	11	194.000	141.000
4	K. L.	F.	Cholelithiasis	5-7	5-7	4^{30}-5	10-17	9-13	7-8	176	22	201.000	204.000
5	Sh. S.	F.	Infectious hepatitis	6-7^{30}	5-6	4-6	11-18	7-9	9-11	33	0	155.000	140.000
6	H. Z.	F.	Postoperative hernia	8^{30}-10	5^{30}-7	6^{30}-7	17-17	10-14	10-15	33	11	182.000	202.000
7	G. E.	F.	Hypert. cardiovasc. dis., posthemiparesis	6^{30}-8^{30}	4^{30}-7	5^{30}-6^{30}	17-22	12-15	13-17	165	88	200.000	180.000
8	B. M.	F.	Colitis ulcerosa, polyposis of colon	5-7^{30}	5-5	5^{30}-7^{30}	15-19	9-13	10-15	11	11	330.000	308.000
9	A. Ch.	M.	West Nile fever	7-8	5-6^{30}	5-7	14-17	9-12	15-18	110	66	300.000	323.000
10	Sh. R.	F.	Rheumatic heart disease, auricular fibrillation, congestive heart failure	5-7	4^{30}-4^{30}	5-6	10-16	9-12	7-10	440	549	301.000	270.000
11	B. I.	M.	Arterioscl. heart disease	6^{30}-8	5^{30}-6	5-5	11-15	11-15	9-10	99	11	206.000	218.000
12	M. M.	M.	Postcholecystectomy, funct. pyloric stenosis	5^{30}-7^{30}	5^{30}-9	6-8^{30}	17-24	16-24	14-19	220	132	295.000	263.000
13	G. R.	F.	Occlusive peripheral vascular disease	6-8^{30}	5-7	5^{30}-8^{30}	11-15	9-12	9-11	121	11	140.000	150.000
14	S. M.	F.	Pyelonephritis	5^{30}-7	5^{30}-7^{30}	5-7	10-15	9-11	8-12	44	11	323.000	318.000
15	K. Sh.	M.	Arterioscl. heart disease, aortic stenosis, congestive heart failure	5-5^{30}	5^{30}-8^{30}	5-6^{30}	11-15	10-15	10-11	143	66	148.000	126.000
16	E. F.	M.	Gastric ulcer, splenomegaly	4^{30}-6	5^{30}-6^{30}	4-5^{30}	12-14	9-12	11-15	154	33	164.000	150.000

17	A. N.	M.	Duodenal ulcer, neurasthenia	5-6^{30}	5-6^{30}	5^{30}-7^{30}	11-15	11-13	11-14	55	55	142.000	170.000
18	E. D.	M.	Nephrolithiasis, pyelonephritis	5-6^{30}	5-7	5^{30}-7	10-14	9-12	12-14	253	132	335.000	330.000
19	E. D.	F.	Essential hypertension	6-9	5^{30}-6^{30}	5^{30}-7^{30}	15-16	16-17	12-15	88	44	210.000	190.000
20	Ch. M.	M.	Anxiety state	6^{30}-9	5-7	6^{30}-9	12-17	11-15	12-17	99	11	278.000	260.000
21	P. Sh.	M.	Postgastrectomy	6-7^{30}	5-7	5^{30}-8	13-15	12-16	11-14	77	0	218.000	190.000
22	P. J.	M.	Amebic colitis, hypertrophy of prostate	4^{30}-6^{30}	5-6^{30}	5-6	11-13	10-13	11-13	77	0	314.000	226.000
23	Ch. S.	F.	Hypertensive cardiovasc.-disease, cong. heart failure	5^{30}-6^{30}	6-8	5^{30}-7^{30}	9-13	11-15	13-17	308	143	240.000	252.000
24	D. R.	F.	Anxiety state, thrombocythemin	5^{30}-7^{30}	7^{30}-8	6-6^{30}	8-12	10-11	8-12	220	11	593.000	452.000
25	L. Ch.	F.	Cystopyelitis	6-6^{30}	5-6	5-6^{30}	11-14	13-18	10-14	143	11	380.000	400.000
26	L. L.	F.	Cholelithiasis				16-18	11-13	16-21	110	11		
27	M. A.	F.	Neurasthenia				13-18	12-16	16-19	220	231		
28	Sh. R.	M.	Multiple myeloma				18-18	9-12	12-17	242	198		
29	A. J.	M.	Cholelithiasis				16-23	11-15		66			
30	K. J.	M.	Myeloid leukemia				19-24	12-17	15-20	77	88		
							5 mg. Prednisone						
31	G. Sh.	M.	Duodenal ulcer	7-9	6^{30}-8	4^{30}-6	18-20	13-17	11-16	143	121	353.000	310.000
							15 mg. Prednisone						
32	N. M.	M.	Duodenal ulcer	6-7^{30}	4^{30}-6	6-7	15-18	9-13	9-12	550	418	180.000	160.000
33	F. J.	M.	Arterioscl. heart dis.	6-8	6-7^{30}	7-9	14-23	8-13	12-15	186	22	219.000	181.000
							20 mg. Prednisone						
34	K. A.	M.	Cooley's trait, "periodic disease"	6-7^{30}	6-7^{30}	6-8^{30}	13-17	11-14	17-20	154	0	252.000	258.000

the few instances where an upward as well as a downward trend appeared in the same experiment, the larger value has been considered as the significant result. Only the values determined in the second tube were used for statistical evaluation. The mean silicone coagulation time before the administration of any of the substances discussed here was 17.21 minutes (151 determinations). In the control group (48 cases) the mean silicone coagulation time before injecting normal saline solution was 16.22 min. ± 0.542 (standard deviation of the mean); after injection it was 16.35 min. ± 0.506.

TABLE IV. STATISTICAL EVALUATION OF THE EFFECTS OF NORMAL SALINE SOLUTION, CORTICOTROPHIN, CORTISONE AND PREDNISONE ON SILICONE COAGULATION TIME IN HUMAN SUBJECTS

SUBSTANCES	MEAN DECREASE OF SILICONE COAG. TIME ± S.D. OF THE MEAN	P			
		CONTROL	CORTICOTROPHIN 25 MG.	CORTICOTROPHIN 50 MG.	CORTISONE
Control normal saline 2 c.c. intramuscular	−0.12 ± 0.37				
Corticotrophin 25 mg. intramuscular	0.30 ± 2.18	$P < 0.9$			
Corticotrophin 50 mg. intramuscular	3.20 ± 0.87	$P < 0.001$	$0.1 < P < 0.2$		
Cortisone 50 mg. intramuscular	2.70 ± 1.02	$0.02 < P < 0.05$	$0.2 < P < 0.3$	$0.9 < P$	
Prednisone 10 mg. oral	4.33 ± 0.79	$P < 0.001$	$0.02 < P < 0.05$	$0.3 < P < 0.4$	$P = 0.2$

All data expressed (in minutes) as the mean decrease ± S.D. of the mean; P = probability factor.

For evaluation of the statistical significance of our results, Table IV has been drawn up. As can be seen from this table, there was hardly any change in silicone coagulation time in the saline injected control group. There was, however, significant shortening of the silicone coagulation time in the other groups. This decrease of coagulation time in the various groups was compared with the control group or the groups themselves. It was found that there was a significant difference among the 50 mg. ACTH, prednisone, and possibly cortisone groups, and the controls, as well as possibly between the prednisone and 25 mg. ACTH group. Thrombocyte fluctuations and outcome of the fibrinogen B tests seem to be insignificant.* The average thrombocyte count, before administration of the various substances in the 61 instances determined, was 232,005 per c.mm., and 224,000 per c.mm. after these substances had been given. Perusal of the tables demonstrates the superiority of the measurement in siliconized tubes over the ones obtained in glass tubes.

*The results are not included in the tables in order to save space.

DISCUSSION

Certain difficulties are bound to arise in an investigation such as the present one when it is intended to study the effect of hormone preparations on blood clotting in hospitalized patients. Ideally, all other factors of possible influence on coagulation time should be safely controlled. It is, however, not surprising that emotional stress cannot always be completely kept out of the experimental period, although we have excluded all tests where such influence was obvious, even before the results of the various determinations had become known. On the other hand, it is the patient in hospital surroundings who is usually treated with the materials investigated here and who will eventually suffer their untoward effects. Under the circumstances, it seems only fair not to expect absolutely constant values for the controls over the 4-hour test period. To our knowledge, the variation in values of coagulation time over the day, particularly with the silicone technique, is not known.

The results of determinations of silicone coagulation time after prednisone were divided into 2 groups; those patients who reacted with a 50 per cent or more decrease of the eosinophils and those who failed to show such a response. This was done in order to determine whether the clotting response would parallel the adrenocortical reaction as evidenced by a significant drop in the absolute eosinophil count. No correlation could be found. The dissociation of these responses or at least the absence of a strict parallelism of these two reactions has been noted before in our study of emotional stress.[19]

In view of other reports on the thrombocyte-increasing effect of corticotrophin, our negative results in these short-term experiments with all 3 substances tested should be noted. Also the fibrinogen B test, except in a very few instances where the patients' illnesses might serve as adequate explanation for its being positive, did not show a response to the hormones tested.

Day-to-day observations of individuals under such hormone treatment might be of importance in order to determine hypercoagulability and a prethrombotic state. However, tentative observations with the aid of the tests used here in patients without such treatment or any medication whatsoever have convinced us of the difficulty of evaluating such studies.

In short-term experiments it is evident that corticotrophin, cortisone, and prednisone in therapeutic doses may occasionally leave the clotting time unaffected, possibly prolong it, or at least not prevent spontaneously occurring prolongation; as a rule, they significantly accelerate whole blood clotting time. The hypercoagulability following the administration of these substances may possibly contribute to the development of thrombosis in susceptible individuals.

SUMMARY

Studies were undertaken in an attempt to determine the influence of corticotrophin and cortisone on the clotting time and thrombocyte counts. Prednisone, not previously studied in this respect, as far as we know, was included in this study.

In short-term experiments extending over 4 hours after administration of these materials and using glass and siliconized tubes, it was found that all 3 substances may leave the clotting time uninfluenced, prolong it slightly (or at least not prevent its spontaneous prolongation) but will usually decrease clotting time appreciably. Experiments carried out after normal saline injections served as controls. Thrombocyte counts remained essentially unchanged, as did the beta naphthol test.

We are greatly indebted to Dr. D. Diengott for his help in the statistical calculations. The help of our technician, Mrs. Miriam Schwartz, is also gratefully acknowledged.

REFERENCES

1. Smith, R. W., Margulis, R. R., Brennan, M. J., and Monto, R. W.: Influence of ACTH and Cortisone on Certain Factors of Blood Coagulation, Science **112**: 295, 1950.
2. de Takats, G., and Voigt, M. T.: The Response of the Clotting Mechanism to ACTH, Angiology **4**: 283, 1953.
3. Cosgriff, S. W., Diefenbach, A. F., and Vogt, W.: Hypercoagulability of the Blood Associated With ACTH and Cortisone Therapy, Am. J. Med. **9**: 752, 1950.
4. Eisenmenger, W. J., Slater, R. J., and Bongiovanni, A. M.: Hypercoagulability of the Blood of Patients With Hepatic Cirrhosis Following Administration of ACTH, Am. J. Med. **13**: 27, 1952.
5. Garrett, J. V.: Heparin and the Adrenal Cortex, J. Clin. Path. **6**: 294, 1953.
6. Bounameaux, Y., van Cauwenberge, H., and Roskam, J.: Le problème des thromboses vasculaires et des hémorrhagies au cours de la thérapeutique cortico-surrénalienne, Schweiz. med. Wchnschr. **84**: 788, 1954.
7. de Nicola, P.: Bemerkungen über die Indikationen und Kontraindikationen der antikoagulanten Therapie auf Grund der gerinnungsphysiologischen Ergebnisse, Schweiz. med. Wchnschr. **84**: 835, 1954.
8. Fahey, J. L.: The Effect of ACTH on Whole Blood Coagulability, Proc. Soc. Exper. Biol. & Med. **77**: 491, 1951.
9. Chevallier, P., Fiebrer, A., and Tadzer, I. S.: Cortisone et coagulation sanguine, Sang **22**: 557, 1951.
10. Sussman, L. N., Cohen, J. B., and Gittler, R.: Clinical Application of Simplified Serum Prothrombin Consumption Test, J. A. M. A. **156**: 702, 1954.
11. Chatterjea, J. B., and Solomon, L.: Antagonistic Effect of ACTH and Cortisone on the Anticoagulant Activity of Ethyl Biscoumacetate, Brit. M. J. **2**: 790, 1954.
12. Fazlullah, S.: Thromboembolic Complications During and After Cortisone and ACTH Therapy, Postgrad. Med. J. **32**: 444, 1956.
13. Koller, F., and Zollikofer, H.: Der Einfluss des adrenocorticotropen Hormons auf die Thrombocytenzahl, Experientia **6**: 299, 1950.
14. Dameshek, W., Saunders, R. H., Jr., and Zannos, L.: The Use of ACTH in the Treatment of Acute and Subacute Leucemia, Bull. New England Med. Center **12**: 11, 1950.
15. Rosenthal, M. C., Saunders, R. H., Schwartz, L. J., Zannos, L., Santiago, E. P., and Dameshek, Wm.: The Use of Adrenocorticotropic Hormone and Cortisone in the Treatment of Leucemia and Leucosarcoma, Blood **6**: 804, 1951.
16. Lauridsen, J., Belko, J. S., and Warren, R.: Alterations in Circulating Platelets Following Administration of Adrenocorticotropic Hormone, Proc. Soc. Exper. Biol. & Med. **79**: 709, 1952.
17. Thorn, G. W., Jenkins, D., Laidlaw, J. C., Goetz, F. C., Dingman, J. F., Arons, W. L., Streaten, D. H. P., and McCracken, B. H.: Pharmacologic Aspects of Adrenocortical Steroids and ACTH in Man, New England J. Med. **248**: 323, 1953.
18. Schneider, R. A.: Recurrent Thrombophlebitis: An Experimental Study of Life Situations and Emotions and the Clotting Time and Relative Viscosity of the Blood, Am. J. Med. Sc. **222**: 562, 1951.
19. Dreyfuss, F.: Coagulation Time of the Blood, Level of Blood Eosinophil and Thrombocytes Under Emotional Stress, J. Psychosom. Res. **1**: 252, 1956.
20. Cummine, H., and Lyons, R. N.: A Study of Intravascular Thrombosis With Some New Corrections of the Mechanism of Coagulation, Brit. J. Surg. **35**: 22, 1948.

STUDIES ON HUMAN SERUM GAMMA GLOBULIN

II. Observations in a Case of Acquired Hemolytic Anemia Due to Cold Autoantibodies, in Multiple Myeloma, and in Other Clinical Problems

Alexander S. Wiener, M.D., Donald K. Briggs, M.D., Leo Weiner, M.D., and Lee Burnett
New York, N. Y.

THERE is considerable clinical interest in the determination of the gamma globulin concentration in the serum. An approximation of the amount of gamma globulin can of course be obtained by electrophoresis, and this technique has become a practicable clinical procedure, with the development of starch and paper electrophoresis. In fact, gamma globulin obtained its name from electrophoresis studies, since by definition it is serum globulin of a certain electrophoretic mobility.

Gamma globulin concentration can also be determined by immunologic methods such as the antiglobulin inhibition test described in previous papers.[1-3] Both electrophoretic and immunologic methods have been successfully used for the diagnosis of agammaglobulinemia,[2, 4] and for following the results of treatment in patients with this disorder receiving injections of gamma globulin. These two methods of estimation complement one another, and the purpose of this paper is to describe how their results can be combined in their application to problems of clinical medicine.

METHODS

The paper electrophoresis technique used for the examination of serum was the standard method using the Spinco apparatus. Standard veronal buffer was used with an ionic strength of 0.075. The current was run for 16 hours at 80 volts and 6 milliamperes per cell. The strips were stained with bromphenol blue and converted to a curve with the photovolt densitometer.

The quantitative inhibition test with antiglobulin immune rabbit serum, which was used for the serologic determination of gamma globulin concentration, has been fully described elsewhere.[1, 2]

RESULTS

1. *Acquired Hemolytic Anemia.*—In a previous paper,[5] an unusual case of acquired hemolytic anemia was described in which the serum of the patient contained an extraordinarily high titer of cold autoantibodies. The titer

From the Serological Laboratory of the Office of the Chief Medical Examiner of New York City, and the Fourth (New York University Post-Graduate Medical School) Medical Division of Bellevue Hospital, New York, N. Y.
Received for publication June 26, 1957.

obtained by serologic titrations gave values of the order of 200,000 units. Results expressed in this way, on the basis of procedures which are crude in comparison with chemical determinations, carry an experimental error which may be as great as 100 per cent or 200 per cent or higher, even in the hands of experienced and careful workers. Moreover, these titer values are arbitrary and depend upon numerous details of technique including the freshness of the test cells, the temperature and duration of incubation, and the criteria used in reading the end point. Nevertheless, if two sera are titrated at the same time by the same worker under identical conditions, it is possible to compare with reasonable accuracy the relative concentrations of the two sera. On this basis, the serum of the patient with cold agglutinins had by far the highest titer of antibody of any case tested by the authors, in an experience over many years with tens of thousands of antisera.

Since antibodies are modified gamma globulins, each arbitrary unit of antibody titer must be equivalent to a certain concentration of antibody gamma globulin in the serum. In fact, one might expect that it should be possible to set up a correlation between antibody titer and gamma globulin concentration in the serum. This has actually been done by Barrett and Tripp[6] for bacterial agglutinins, and a table for red cell agglutinins has been published in a previous paper.[7] In this table, the arbitrary unit of titer is assumed to represent 0.7 μg of gamma globulin per milliliter of serum. On this basis, if one assumes that normal serum contains 1.2 Gm. of gamma globulin per 100 ml., and that all of it were converted into hemagglutinin, the titer of the latter would be expected to be about 16,000 units. This, accordingly, sets an upper limit to the hemagglutinin titer, which is in conformity with our experience. These considerations cast a measure of doubt on certain reports in the literature which mention titers as high as 28,000,000 units, since this would imply concentrations of gamma globulin of the order of 20 Gm. per milliliter of serum.

In the unique patient described by us,[5] the observed titer in the neighborhood of 200,000 units would suggest a considerably elevated concentration of gamma globulin in the serum, even assuming that all the gamma globulin had converted to antibody. In this instance it was not possible to employ the gamma globulin inhibition technique, since the serum would have agglutinated the test cells directly. Hence, it was necessary to rely upon electrophoresis, which demonstrated the amount of protein present in the globulin fraction, even though this technique gave no indication of its immunologic activity.

Fig. 1 shows the pattern obtained on electrophoretic analysis of this patient's serum, which had a total protein concentration of 6.5 Gm. per 100 ml. It will be seen that, as expected, there is a very high and wide peak in the gamma globulin fraction, which exceeds the peak corresponding to the albumin fraction of the serum. The bifid shape of the gamma globulin peak is perhaps due to an artifact. When the same serum was studied by Dr. H. Fudenberg and Dr. H. G. Kunkel,[8] using the method of starch electrophoresis, a single peak was obtained in the gamma globulin band, which was approximately

twice as high as the albumin peak and almost twice as wide. By zone electrophoresis, these workers showed that the serologic activity of the serum was confined to the peak in the gamma globulin region.

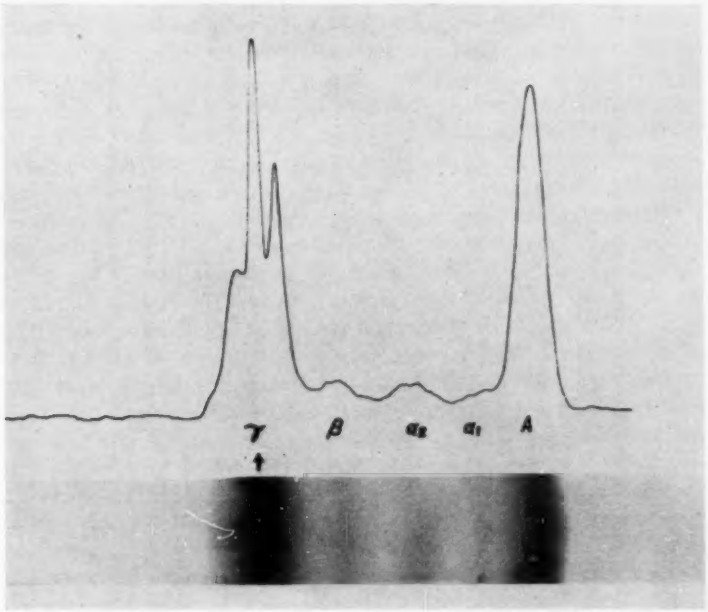


Fig. 1.—Electrophoretic pattern of serum from a patient with acquired hemolytic anemia, and having a very high titer of cold autoagglutinins in her serum.

2. *Multiple Myeloma.*—In classical cases of multiple myeloma, the bone marrow is pervaded with abnormal plasmacytes and the serum contains a high concentration of protein because of an excess of globulin. Many studies have been attempted to determine the nature of the serum globulin responsible for the classical findings of multiple myeloma, and to indicate whether it represents an excess of a normal serum constituent or is in fact an abnormal protein specific for the disease. The consensus, based on experimental studies, suggests that an abnormal protein is produced by the pathologic plasmacytes of the bone marrow. Some investigators[9] report that, by immunization of rabbits with serum from patients with myeloma they have been able to produce reagents which will precipitate serum from patients with myeloma, but not normal serum. Unfortunately, such specific serologic reagents are not easy to prepare, so that the method is not readily applicable as a diagnostic test.

A number of patients with myeloma have serum giving an electrophoretic pattern with a peak at a point which does not correspond to any of the normal protein constituents, while others merely show what appears as a marked accentuation of the gamma globulin band. The question arises whether the latter represents an increase in normal gamma globulin, or the presence of a superimposed abnormal protein of the same electrophoretic mobility as gamma

TABLE I. GAMMA GLOBULIN CONCENTRATION IN THE BLOOD SERUM OF PATIENTS WITH MYELOMA, AS DETERMINED BY THE INHIBITION TEST*

CASE NUMBER	DIAGNOSIS	RESULTS OF INHIBITION TESTS			CHEMICAL ESTIMATION OF PROTEIN		ABO GROUP	ISOAG-GLUTININ TITER	RESULTS OF ELECTROPHORESIS
		INHIBITION TITER OF NORMAL SERUM (UNITS)	INHIBITION TITER OF PATIENT'S SERUM (UNITS)	GAMMA GLOBULIN CONCENTRATION AS PERCENTAGE OF NORMAL	ALB. (GM./100 ML.)	GLOB. (GM./100 ML.)			
1. C. B.	Myeloma	560	220	40	3.7	7.0	A	Low	Heavy gamma band. Gamma globulin in urine
2. L. L.	Myeloma	560	80	14	4.3	3.4	O	Low	Heavy gamma band
3. S. P.	Myeloma	560	80	14	4.4	3.4	O	Very low	Heavy gamma band
4. A. W.	Myeloma	280	85	30	6.2	1.3	A	Zero	M myeloma band†
5. L. A.‡	Myeloma	120	120	100	Unavailable		O	Below average	Not done
6. J. G.	Chronic lymphatic leukemia	120	4	3.3	4.0	1.9	A	Zero	Absent gamma globulin

*Included in table for comparison is one case of chronic lymphatic leukemia, with acquired agammaglobulinemia.

†Band between beta and gamma; also positive reactions for Bence-Jones protein.

‡This patient was transferred to another hospital and the records are no longer available. Under these circumstances the diagnosis of myeloma remains dubious.

globulin. In an attempt to answer this question a series of patients with myeloma was studied by the gamma globulin inhibition method, as well as by electrophoresis.

In Table I are presented results of studies on 5 such patients as well as one patient with chronic lymphatic leukemia, included for comparison. As can be seen, one patient (Case 4) had an abnormal globulin in the serum as demonstrated by electrophoresis, namely, the so-called "M" myeloma protein characterized by a peak between beta and gamma in the electrophoretic pattern. This patient excreted a good deal of the M protein in the urine, as well as some Bence-Jones protein. The antiglobulin inhibition test showed that the serum contained approximately 30 per cent of the normal concentration of gamma globulin. Three patients had a heavy gamma band in the electrophoretic pattern, yet by the inhibition test the gamma globulin was found to range from 14 to 40 per cent of the normal. This indicates that the protein responsible for the heavy gamma band is most likely an abnormal one incapable of functioning in the same way as normal gamma globulin with regard to the formation of antibodies, despite its similar electrophoretic mobility. In support of this interpretation may be cited the report by Lawson and coworkers[10] that complete absence of antibodies or deficiency of these substances is a characteristic feature of myelomatosis. (The case of chronic lymphatic leukemia is included in Table I for comparison, and is of interest because of the almost complete absence of gamma globulin. This is a not uncommon finding in chronic lymphatic leukemia, but is by no means characteristic of the disease.)

It may be of interest to mention in passing that recently we immunized rabbits with serum from a patient with agammaglobulinemia. The rabbit serum was absorbed to remove immune agglutinins and then used as a reagent for the antiglobulin test and found to give as potent reactions as similar reagents prepared from immune rabbit sera for normal human serum. It is known that in so-called agammaglobulinemia, gamma globulin is actually present in the plasma in low concentration, of the order of 20 to 40 mg./100 ml. Thus, our observations indicate that even very small amounts of human gamma globulin can stimulate the production of specific antibodies in the rabbit.

3. *Serum Gamma Globulin Concentration in Cases of Sudden Death in Infancy.*—Electrophoretic studies have shown that in newborn infants the gamma globulin concentration is approximately the same as in the mother, and that after birth the gamma globulin concentration decreases, reaching a minimum at 3 months, after which it increases.[11, 12] These findings parallel observations on antibody in newborn babies, since it has been found that the antibodies present at birth are passively acquired by placental transfer from the mother. The passively acquired antibody molecules have a half-life of 30 days,[13] so that these gradually disappear from the baby's circulation, and later on the baby produces his own antibodies in response to suitable antigenic stimuli.

These observations may help to throw some light on certain unexplained deaths during the neonatal period. Such deaths have frequently been attributed to suffocation of weak infants by heavy bedclothes, pillows and the

like. Dr. J. Werne for many years maintained that the normal infant would strongly resist such suffocation, and believed that these sudden deaths were due to infections to which the baby had not developed immunity. In fact, for a period of time he submitted tissues from such medicolegal cases to one of us (A. S. W.) for bacteriologic examination, but, unfortunately, the results of these studies were inconclusive.

An observation which tends to support the thesis that unexplained death in infants may be due to unrecognized infections is that such deaths tend to occur at that period in neonatal life when gamma globulin concentration in the blood plasma is at its lowest point. In Table II is presented a series of such cases of unexpected death in infants, in which serum samples were submitted by Dr. William Benenson for gamma globulin determinations. As shown in the table, the results of the inhibition test showed the gamma globulin concentration to range from 8.5 to 37 per cent of the adult normal in 11 of the 12 cases. In only one of the cases was the concentration as high as 60 per cent of the normal adult value. Moreover, all but one of the babies were within the critical age period of 2 to 5 months.

TABLE II. SERUM GAMMA GLOBULIN CONCENTRATION IN CASES OF SUDDEN UNEXPLAINED DEATH IN INFANCY*

PATIENT AND CASE NUMBER	AGE OF INFANT	RESULTS OF INHIBITION TESTS		
		INHIBITION TITER OF NORMAL ADULT SERUM	INHIBITION TITER OF INFANT SERUM	GAMMA GLOBULIN CONCENTRATION EXPRESSED AS PERCENTAGE OF NORMAL
1. J. DiP. (16)	2 mo.	48	7	14
2. M. (60)	3 mo.	200	50	25
3. S. M. (55)	2 mo.	200	60	30
4. D. S. (56)	2 mo.	200	40	20
5. S.	2½ yr.	320	120	37
6. B.	5 mo.	320	80	25
7. A.	2 mo.	640	380	60
8. K. R.	4 mo.	120	40	33
9. K. McN.	4 mo.	120	10	8.5
10. P. D. (76)	2 mo.	60	15	25
11. G. N. (84)	3 mo.	60	20	33
12. S. W. (82)	4 mo.	60	15	25

*The sera of these patients were made available to us by Dr. William Benenson of the Office of the Chief Medical Examiner of N. Y. C., County of Queens.

Of course it is realized that the demonstration of low gamma globulin in these cases of unexpected death in infancy does not necessarily establish a causal relationship, since innumerable babies with such low gamma globulin values fail to develop infections.

4. *Splenectomized Patients.*—Because of the reputed role of the spleen in immunity, the serum gamma globulin concentration was determined by electrophoresis and by the inhibition technique in two patients who had been splenectomized 5 years previously; one was a boy with Cooley's anemia, the other a woman with acquired hemolytic anemia. In both, normal concentrations of serum gamma globulin were found, in line with the results of immunization experiments on splenectomized individuals by Myerson and associates.[14]

SUMMARY

An account is given of clinical experiences with the application of the previously described antiglobulin inhibition test for the estimation of serum gamma globulin.

The presence of approximately 4 times the normal amount of gamma globulin was demonstrated by electrophoresis in a case of acquired hemolytic anemia. (The hemolytic anemia was caused by potent cold autoantibodies which interfered with the application of the antiglobulin inhibition test.) Presumably almost all of this gamma globulin represented cold autoagglutinins.

The antiglobulin inhibition test demonstrated a diminution of "functional" gamma globulin in some cases of myeloma, where electrophoresis suggested an increase in this fraction of the serum proteins.

The serologic and electrophoretic tests agreed in confirming the diagnosis in a case of agammaglobulinemia, and in demonstrating the relatively low serum gamma globulin levels of early infancy. In two splenectomized patients, the two methods likewise agreed in showing normal concentrations of serum gamma globulin.

Thus, the two methods, electrophoresis and antiglobulin inhibition, complement one another, and both may be required for the full investigation of a patient.

REFERENCES

1. Wiener, A. S., Hyman, M. A., and Handman, L.: A New Serologic Test (Inhibition Test) for Human Serum Globulin, Proc. Soc. Exper. Biol. & Med. **71**: 96-99, 1949.
2. Wiener, A. S.: Serologic Test for Human Gamma Globulin. II. Application in Diagnosis and Treatment of Agammaglobulinemia, Am. J. Clin. Path. **25**: 595-597, 1955.
3. Wiener, A. S., and Gordon, E. B.: Studies on Human Serum Gamma Globulin. I. The Half-Life and Rate of Production, J. Lab. & Clin. Med. **49**: 258-262, 1957.
4. Good, R. A., and Zack, S. J.: Disturbances in Gamma Globulin Synthesis as "Experiments of Nature," Pediatrics **18**: 109-148, 1956.
5. Wiener, A. S., Unger, L. J., Cohen, L., and Feldman, J.: Type-Specific Cold Auto-Antibodies as a Cause of Acquired Hemolytic Anemia and Hemolytic Transfusion Reactions; Biologic Test with Bovine Red Cells, Ann. Int. Med. **44**: 221-240, 1956.
6. Barrett, C. D., Jr., and Tripp, J. A.: A Method of Adjusting the Antigen Antibody Ratio Prior to the Determination of Pneumococcic Antibody Nitrogen, J. Immunol. **43**: 311-317, 1942.
7. Wiener, A. S., Wexler, I. B., and Hurst, J. G.: The Use of Exchange Transfusion for the Treatment of Severe Erythroblastosis Due to A-B Sensitization, With Observations on the Pathogenesis of the Disease, Blood **4**: 537-555, 1949.
8. Fudenberg, H., and Kunkel, H. G.: Physical Properties of the Red Cell Agglutinins in Acquired Hemolytic Anemia, J. Exper. Med. **106**: 689-702, 1957.
9. Collier, F. C., and Jackson, P.: The Precipitin Test for Bence-Jones Protein, New England J. Med. **248**: 409-414, 1953.
10. Lawson, H. A., Stuart, C. A., Paull, A. M., Phillips, A. M., and Phillips, R. W.: Observations on the Antibody Content of Blood in Patients With Multiple Myeloma, New England J. Med. **252**: 13-18, 1955.
11. Wiener, A. S., Berger, H., and Lenke, S.: Serum Gamma Globulin in Infants, Lab. Digest **14**: 11-12, 1951.
12. Orlandini, O., Sass-Kartsak, A., and Ebbs, J. H.: Serum Gamma Globulin Levels in Normal Infants, Pediatrics **16**: 575-584, 1955.
13. Wiener, A. S.: The Half-Life of Passively Acquired Antibody Globulin Molecules in Infants, J. Exper. Med. **94**: 213-221, 1951.
14. Myerson, R. M., Stout, R., and Haven, W. P.: The Production of Antibody by Splenectomized Persons, Am. J. M. Sc. **234**: 297-300, 1957.

THE INFLUENCE OF PERITONEAL PUNCTURE WITH AND WITHOUT INTRAPERITONEAL AIR INSUFFLATION ON RENAL FUNCTION AND ELECTROLYTE EXCRETION

NEAL S. BRICKER, M.D.,* AND LLOYD GREGORY, JR., M.D.
ST. LOUIS, MO., AND DENVER, COLO.

MUCH attention has recently been focused upon reproducible experimental states which are characterized by acute reductions of the excretion rates of sodium chloride and water. A variety of stimuli have been found to evoke these changes, including (1) constriction of the renal vein in dogs[1]; (2) quiet standing in normal humans[2]; (3) inflation of blood pressure cuffs about the extremities of supine subjects[3]; (4) inflation of a balloon in both inferior and superior vena cavae of humans[4]; and (5) increasing intra-abdominal pressure by inflation of a pneumatic girdle about the abdomen of normal human subjects.[5] The results of such experiments have been examined both for information regarding the factors influencing sodium chloride excretion in the normal state as well as for possible insight into mechanisms operative in edema-forming states. The majority of these states has been associated with the induction of an increase in systemic and/or renal venous pressure or with a decrease in circulating blood volume, and certain of the data have served as important evidence in developing the concept of a volume control system.

In the present study acute reductions in the excretion rates of salt and water have been evoked by the induction of pneumoperitoneum and by peritoneal puncture without intraperitoneal air insufflation. Neither of the procedures involves direct alterations of pressures, volumes, or distribution of body fluids.

METHODS

Subjects.—Renal clearance studies were performed on 9 patients with asymptomatic quiescent or regressing pulmonary tuberculosis. None of the patients demonstrated evidence of disseminated (including renal) involvement, and none was hyponatremic. All subjects were on an unrestricted diet and had been treated for at least 4 months prior to study with bed rest and at least 2 of 3 drugs including streptomycin, para-aminosalicylic acid, and isonicotinic acid hydrazide. All drugs were withheld for 24 and in the majority of instances for 72 hours prior to study. The ages for the group ranged from 20 to 43 with a mean of 28 years.

Procedure.—The patients were studied in the morning, in the recumbent position and in the fasting state. A liter of water was ingested approximately 90 minutes prior to the intravenous administration of the priming solution, and additional water in 125 ml. quantities was administered every 30 to 45 minutes throughout the duration of measurements.

From the Renal Physiology Section, Research and Development Branch, Fitzsimons Army Hospital, Denver, Colo.

Presented in part at the National Meeting of the American Federation for Clinical Research, Atlantic City, N. J., May, 1954.

Received for publication Sept. 14, 1957.

*Present address: Washington University School of Medicine, St. Louis, Mo.

Urine was collected through an inlying soft multiholed catheter, and the bladder was washed with two separate 10 ml. aliquots of distilled water and two or more injections of air.

After a timed collection of urine for determination of any blank excretion of inuloid or PAH-like substances, a priming injection of inulin and PAH was administered and a sustaining solution of the same substances initiated. The latter was delivered in an isotonic saline vehicle at 1.5 ml./ min. using a Bowman constant infusion pump. Plasma concentrations of inulin were maintained at approximately 25 mg. per cent and PAH at approximately 2 mg. per cent. A period of 45 minutes was allowed for establishment of equilibrium of inulin and PAH, following which 3 *control* clearance periods were obtained. The experimental procedure was then concluded and an additional 10 to 20 minutes allowed to compensate for estimated "nephron delay time." Thereafter 3 *experimental* clearance periods were obtained. The duration of individual clearance periods ranged from 15 to 20 minutes.

The experimental procedure consisted of anesthetizing a 2 by 2 cm. area of skin, subcutaneous tissue, and peritoneum with 2 per cent Metycaine solution and introducing an 18 gauge needle into the anesthetized area and through the peritoneum. In 5 subjects a liter of air was administered intraperitoneally; in 3 the needle was inadvertently withdrawn slightly and the majority of air deposited subcutaneously; and in one no air was administered. In one of the patients studied during initial pneuoperitoneum, clearances were repeated after 3 months of weekly pneumoperitoneum refills. The final intra-abdominal pressure in those subjects receiving intraperitoneal air insufflation approximated 10 cm. of water.

Venous blood samples were drawn (and centrifuged without delay) at the mid-point of each clearance period through an inlying 17 gauge thin-walled needle. Approximately 0.1 ml. of heparin was used for each 10 ml. of blood. The inlying needle was maintained patent by injection of 0.3 ml. of a dilute heparin solution after each blood sampling. Prior to the subsequent sampling, 2 ml. of blood were withdrawn in a separate syringe and discarded to prevent dilution of the sample used for analyses.

Inulin was determined according to the method of Roe, Epstein, and Goldstein[6]; PAH, according to the method of Smith and associates[7]; sodium and potassium on a Baird internally compensated flame photometer; and chloride according to Van Slyke and Hiller's modification of Sendroy's iodometric titration technique.[8]

RESULTS

Peritoneal Puncture With Intraperitoneal Air Injection.—Following deposition of local Metycaine, puncture of the peritoneum, and insufflation of approximately a liter of air intraperitoneally in 5 patients, a characteristic response was noted (Table I, Cases 1 to 5). The results in a representative case are shown in Fig. 1. Inulin clearance (C_{in}) decreased slightly in all patients, ranging from 2 to 16 per cent.* PAH clearance (C_{PAH}) also decreased consistently (7 to 16 per cent), however the relative decreases in glomerular filtration rate and renal plasma flow varied somewhat from patient to patient such that filtration fractions decreased in 2 subjects and increased in 3. Chloride excretion rates decreased from 30 to 60 per cent in 4 of the 5 experiments. In one patient there was a 10 per cent rise in chloride excretion rate. Sodium excretion rates similarly decreased in 4 subjects (21 to 36 per cent) and increased in 1 (9 per cent). All 5 patients exhibited a decrease in potassium

*Values represent percentage change between mean values of control versus mean values of experimental periods.

TABLE I. EFFECTS OF EXPERIMENTAL PROCEDURE ON GFR, ERPF, AND THE EXCRETION RATES OF Cl, Na, AND K

CASE	AGE	EXPERIMENTAL PROCEDURE*	GFR ML./MIN./1.73 M.²			ERPF ML./MIN./1.73 M.²			Cl EXCRETION RATE μEq/MIN.			Na EXCRETION RATE μEq/MIN.			K EXCRETION RATE μEq/MIN.		
			MEAN PER. 1-3	MEAN PER. 4-6	PER CENT CHANGE	MEAN PER. 1-3	MEAN PER. 4-6	PER CENT CHANGE	MEAN PER. 1-3	MEAN PER. 4-6	PER CENT CHANGE	MEAN PER. 1-3	MEAN PER. 4-6	PER CENT CHANGE	MEAN PER. 1-3	MEAN PER. 4-6	PER CENT CHANGE
1	21	PNP	112.2	102.5	- 8.6	639.0	591.0	- 7.4	259.7	181.0	-30.3	311.0	229.0	-26.4	57.2	22.8	-60.8
2	35	PNP	137.3	128.1	- 6.7	593.0	523.0	-11.8	379.3	246.0	-35.1	385.3	272.7	-29.2	79.9	42.8	-46.4
3	32	PNP	86.4	85.1	- 1.6	692.0	581.0	-16.0	163.3	65.4	-60.0	203.3	130.0	-36.1	77.6	58.2	-25.0
4	32	PNP	83.0	79.8	- 3.9	409.0	347.0	-15.3	301.7	195.0	-35.4	344.7	271.5	-21.2	87.8	47.8	-45.6
5	22	PNP	85.7	70.9	-16.1	498.0	444.0	-10.8	82.7	91.3	+10.4	108.3	118.3	+ 9.2	64.3	40.9	-36.4
6	21	PP (Sub. Air)	132.0	120.0	- 9.1	633.0	594.0	- 6.3	154.7	71.6	-53.7	191.7	97.9	-49.1	108.7	74.7	-31.1
7	43	PP (Sub. Air)	112.0	125.0	+11.6	601.0	663.0	+10.9	185.3	90.1	-51.4	295.3	177.0	-40.1	78.0	45.9	-41.2
8	31	PP (Sub. Air)	107.0	119.0	+11.2	557.0	595.0	+ 6.8	269.3	191.5	-28.9	287.3	205.0	-28.6	55.4	56.0	+ 1.1
9	20	PP	126.0	119.0	- 5.6	658.0	538.0	-18.2	209.0	104.1	-50.1	170.3	108.7	-36.2	132.0	88.3	-33.1

The experimental procedure was initiated after the conclusion of period 3 and completed 10 to 20 minutes before the beginning of period 4. Individual periods varied from 15 to 20 minutes in duration.

ERPF = Effective renal plasma flow.

*PNP = Peritoneal puncture with intraperitoneal air insufflation; PP (Sub. Air) = peritoneal puncture with subcutaneous air insufflation; PP = peritoneal puncture without air insufflation.

excretion rates ranging from 25 to 61 per cent. Urine flow rates decreased markedly in all patients and generally were less than 1 ml. per minute during the experimental periods.

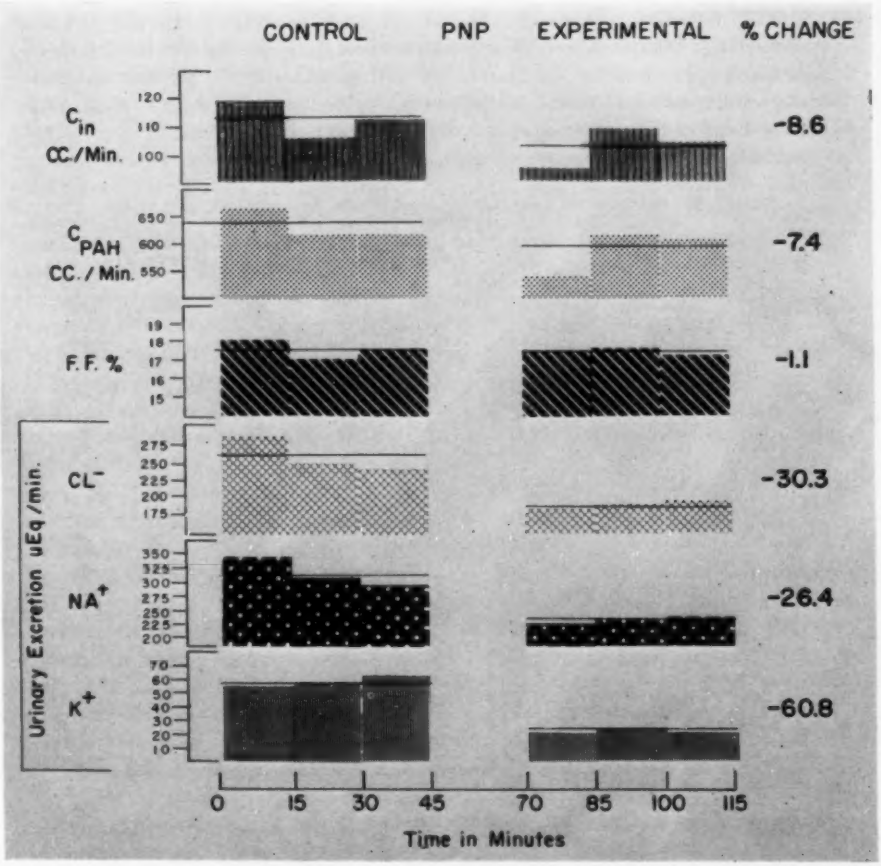


Fig. 1.—Effects of pneumoperitoneum on renal hemodynamics and electrolyte excretion in a representative case. (PNP = pneumoperitoneum.)

Peritoneal Puncture With Subcutaneous Air Injection.—In the 3 patients in whom air was deposited subcutaneously (Table I, Cases 6 to 8), C_{in} and C_{PAH} decreased in 1 and increased in 2. Filtration fractions again showed no consistent pattern. All 3 subjects sustained decreases in the excretion rates of chloride and sodium. Potassium excretion rates decreased in 2 and remained essentially unchanged in 1. Urine flow decreased precipitously in each case.

Peritoneal Puncture Without Air Administration.—One patient was studied before and after peritoneal puncture without the administration of air (Table

I, Case 9). The experimental conditions were otherwise identical to those employed in the foregoing cases. Inulin clearance decreased 6 per cent and C_{PAH} decreased 18 per cent. Chloride excretion decreased 50 per cent, sodium excretion rate 36 per cent potassium excretion rate 33 per cent, and a marked antidiuresis occurred.

Follow-Up Study.—One subject was studied both during the induction of initial pneumoperitoneum and again after 3 months of weekly pneumoperitoneum refills. The response to the initial procedure (Table I, Case 4) was typical. During the follow-up procedure, however, the characteristic changes did not occur.

TABLE II. STATISTICAL ANALYSIS OF DATA FROM ALL SUBJECTS RECEIVING INITIAL PERITONEAL PUNCTURE (CASES 1 THROUGH 9)

FUNCTION		CONTROL PERIODS			EXPERIMENTAL PERIODS		
		1	2	3	4	5	6
Glomerular filtration rate	Mean value	110	107	110	103	109	106
	Composite control mean	109	109	109	109	112	109
	P value	0.9	0.7	0.9	0.2	0.6	0.6
Effective renal plasma flow	Mean value	603	576	581	521	564	563
	Composite control mean	587	587	587	587	609	590
	P value	0.25	0.25	0.7	0.02	0.13	0.2
Cl excretion rate	Mean value	238	214	216	137	124	135
	Composite control mean	223	223	223	223	213	217
	P value	0.1	0.05	0.25	0.001	0.01	0.01
Na excretion rate	Mean value	267	245	254	176	161	194
	Composite control mean	255	255	255	255	244	258
	P value	0.1	0.05	0.9	0.001	0.01	0.2
K excretion rate	Mean value	84.1	83.3	79.7	61.6	50.0	45.6
	Composite control mean	82.4	82.4	82.4	82.4	81.7	86.9
	P value	0.7	0.7	0.4	0.01	0.001	0.001

Mean values for each individual clearance period are compared to the composite mean for the 3 control periods. When a value for any function was not available for a given experimental period, the corresponding control values were omitted from the calculation of the composite control means.

Statistical Analysis.—Analysis of the composite data on the 9 subjects receiving initial peritoneal puncture, with or without air, revealed decreases in the mean values after the experimental procedure for all parameters studied. The changes for the group were as follows: C_{in}, 3 per cent; C_{PAH}, 9 per cent; chloride excretion rate, 37 per cent; sodium excretion rate, 29 per cent; and potassium excretion rate, 35 per cent. Analysis of the data for each individual clearance period is included in Table II. The changes in C_{in} were not statistically significant (P = 0.2 or greater) in all 3 experimental periods and the changes in C_{PAH} were not significant (P = > 0.1) in 2 of the 3 experimental periods. The changes for all 3 electrolytes were of a high order of significance (P = 0.01 or less) in at least 2 of the 3 experimental periods.

DISCUSSION

The present observations indicate that puncture of the peritoneum with or without insufflation of air intraperitoneally in recumbent human subjects is attended by (1) a profound antidiuresis, and (2) an abrupt and sustained decrease in the excretion rates of Cl, Na, and K. The antidiuresis may probably be related primarily to a release of antidiuretic hormone evoked by the experimental procedure. The reabsorption of a proportionately greater amount of water than electrolytes is indicated by the fact that the concentrations of sodium and chloride in the urine generally increased despite the decrease in the excretion rates of these ions. The mechanisms responsible for the decreased electrolyte excretion rates are poorly defined. The changes could have been due predominantly to either (a) a decrease in filtered load of electrolytes secondary to a drop in glomerular filtration rate, or (b) an alteration of the rate of tubular transport of sodium and chloride. Although the decreases in electrolyte excretion rates are statistically significant whereas the changes in glomerular filtration rates are not, this does not permit the conclusion that the primary alterations were in tubular transport. Thus in 6 of the 9 studies, the changes in filtration rate were of sufficient magnitude to account for the decreased electrolyte excretion rates. In the other studies the possibility cannot be excluded that a decrease in filtered load was obscured by the inherent error in the inulin clearance procedure. Although it is well established that increasing intra-abdominal pressure in both animals and man will influence urine flow,[9] renal hemodynamics,[10] and electrolyte excretion rates,[5] the intra-abdominal pressures employed to effect these changes have generally exceeded 15 mm. Hg. The intra-abdominal pressures in the present patients receiving intraperitoneal air did not exceed 7 mm. Hg. Moreover in the 3 patients who received the air subcutaneously and in the subject who received no air, the changes in intra-abdominal pressure were negligible. It seems unlikely, therefore, that the observed effects represented a response to a mechanical increase in renal venous pressure. There is also nothing to suggest that the experimental procedure per se mechanically induced the pooling of blood or the elevation of venous pressure in either the superior or inferior vena cava.

The variety of stimuli which have been found by previous workers to evoke acute salt and water retention by the kidneys, suggests an element of nonspecificity to the reaction. If such changes occur as a regulatory function of the still hypothetic volume control system, the present data would imply that factors other than alterations of volume of some component of body fluids may initiate identical changes.

SUMMARY

1. It has been noted that peritoneal puncture with insufflation of approximately a liter of air intraperitoneally induced acute and significant decreases in the excretion rates of chloride, sodium, and potassium.

2. The same alterations of renal excretion occurred with peritoneal puncture and insufflation of the air subcutaneously and in one case with peritoneal puncture without insufflation of any air.

3. Antidiuresis consistently occurred immediately after peritoneal puncture and was provisionally attribued to antidiuretic hormone release secondary to painful stimuli.

4. It is considered unlikely that the procedures employed mechanically increased renal or vena caval venous pressure, or decreased effective circulating blood volume.

5. It is concluded that if changes in electrolyte and water excretion, such as those noted in the present study, are mediated by a volume control system, factors other than alteration of volume of some component of body fluids may initiate an identical response.

REFERENCES

1. Blake, W. D., Wegria, R., Keating, R. P., and Ward, H. P.: Effect of Increased Renal Venous Pressure on Renal Function, Am. J. Physiol. **157**: 1, 1949.
2. Epstein, F. H., Goodyear, A., Lawrason, F., and Relman, A. S.: Studies of the Antidiuresis of Quiet Standing: The Importance of Changes in Plasma Volume and Glomerular Filtration Rate, J. Clin. Invest. **30**: 63, 1951.
3. Fitzhugh, F. W., McWhorter, R. L., Estes, E. H., Warren, J. V., and Merrill, A. J.: The Effect of Application of Tourniquets to the Legs on Cardiac Output and Renal Function in Normal Human Subjects, J. Clin. Invest. **32**: 1163, 1953.
4. Farber, S. J., Becker, W. H., and Eichna, L. W.: Electrolyte and Water Excretions and Renal Hemodynamics During Induced Congestion of the Superior and Inferior Vena Cava of Man, J. Clin. Invest. **32**: 1145, 1953.
5. Bradley, S. E., Mudge, G. H., Blake, D. W., and Alphonse, P.: The Effect of Increased Intra-Abdominal Pressure Upon Renal Excretion of Water, Sodium and Potassium in Normal Human Subjects and in Patients With Diabetes Insipidus, J. Clin. Invest. **28**: 772, 1949.
6. Roe, J. H., Epstein, J. H., and Goldstein, N. P.: A Photometric Method for the Determination of Inulin in Plasma and Urine, J. Biol. Chem. **178**: 839, 1949.
7. Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M.: The Renal Clearance of Substituted Hippuric Acid Derivatives and Other Aromatic Acids in Dogs and Man, J. Clin. Invest. **24**: 388, 1945.
8. Van Slyke, D. D., and Hiller, A.: Application of Sendroy's Iodometric Chloride Titration to Protein-Containing Fluids, J. Biol Chem. **167**: 107, 1947.
9. Thorington, J. M., and Schmidt, C. F.: A Study of Urinary Output and Blood Pressure Changes Resulting in Experimental Ascites, Am. J. M. Sc. **165**: 880, 1923.
10. Bradley, S. E., and Bradley, G. P.: The Effect of Increased Intra-Abdominal Pressure on Renal Function in Man, J. Clin. Invest. **26**: 1010, 1947.

THE RELATIONSHIP BETWEEN ALVEOLAR AND BLOOD CARBON MONOXIDE CONCENTRATIONS DURING BREATHHOLDING

Simple Estimation of COHb Saturation

Robert H. Jones, M.D.,* Marjorie F. Ellicott, M.D., A.M., John B. Cadigan, M.D.,* and Edward A. Gaensler, M.D.,**
Boston, Mass.

IN HOSPITALS the diagnosis of carbon monoxide poisoning usually is based on history and physical findings and can be confirmed by blood analysis. In industries where exposure to carbon monoxide is a hazard, protection by automatic alarming devices is common. In many situations, however, monitoring of inspired gas is either not practicable or, if feasible, only of limited value. Analysis of ambient air may fail to give a true index of exposure: the relation of environmental carbon monoxide (CO) concentration to the carboxyhemoglobin (COHb) saturation varies with length of exposure, with rate of basal metabolic activity, with intensity and duration of exercise,[1, 2] with concentration of blood hemoglobin, and with partial pressure of oxygen in the ambient air. Even if these factors are considered, the COHb level may be underestimated by as much as 10 per cent saturation by neglecting the possible effect of cigarette smoking.

Reliance on symptoms for estimation of COHb levels is likewise of limited value: exposure to high concentrations of CO may lead to coma without prior warning, while mild symptoms such as dizziness, headache, weakness, fatigue, and shortness of breath are not specific for CO poisoning.

For these reasons a need was felt for a rapid, simple, and precise method for direct determination of COHb saturation. First, a technique was developed for accurate measurement of CO content in whole blood. This involved vacuum extraction of gas and CO analysis by a physical method after dilution of the sample to a known volume.[3] However, the method required venipuncture, manometric analytical apparatus, and a trained technician and, therefore, was not suitable for routine examination of exposed personnel in the field.

From the Thorndike Memorial Laboratory and Second and Fourth (Harvard) Medical Services, the Sears Surgical Laboratory and Fifth (Harvard) Surgical Service, and the Eighth (Thoracic) Surgical Service, Boston City Hospital; the Physiology Laboratory, Sanatorium Division of the Boston City Hospital; the Departments of Medicine and Surgery, Harvard Medical School; and the Department of Surgery, Boston University School of Medicine.

This study was supported, in part, by research grants from the National Heart Institute, United States Public Health Service (H-1773) and from the Committee on Medical Research of the American Trudeau Society, medical section of the National Tuberculosis Association.

Received for publication Sept. 20, 1957.

*U. S. Public Health Service Research Fellow.

**American Trudeau Society Fellow.

In this report is described another method which obviates these shortcomings. Its principle involves use of the lungs as aerotonometers until gas pressures of pulmonary blood and alveoli approach equilibrium.[4-6] The COHb per cent saturation is then estimated by rapid physical analysis of the CO content of expired air with a simple instrument generally available to industry.

METHOD

The subjects studied were 10 physicians and technicians between the ages of 21 and 36. After some experimentation, to be described under "Results," the following technique was adopted for alveolar sampling: the subject was instructed to exhale completely, inhale rapidly and maximally, hold his breath for 20 seconds and then exhale rapidly through one orifice of a large-bore, three-way aluminum valve. The second orifice of this valve was open to room air and to the third was attached a two-liter anesthesia bag (with nipple) previously emptied by wringing. An end-expiratory sample of about 500 ml. was collected in the bag. The dead space of the valve caused an insignificant error. The gas was analyzed for CO_2 with a previously standardized infrared meter,* and for CO with another special infrared meter† which was modified for highest accuracy.[3] The standard gas used for these tests was 0.025 per cent CO; and the zero setting was obtained with room air freed of CO by a hopcalite‡ filter. The response of the CO meter to known concentrations of CO_2 and the preparation of the standard gas have been previously described.[3] The effect of alveolar CO_2 on the CO meter reading was calculated from the known CO_2 concentration and the reading of the CO meter was corrected accordingly. The partial pressure of CO in the alveolar gas (pCO) was calculated by multiplying the corrected CO concentration by the barometric pressure minus 47 mm. Hg.

Venous blood was drawn at the time of alveolar sampling and analyzed for CO content by the method of Gaensler and associates.[3] The blood CO capacity was calculated from two sources: the photometrically determined hemoglobin and the oxygen capacity determined by the usual Van Slyke-Neill technique. However, because the CO content of the blood interfered with O_2 capacity determination, the photometric method was found more reliable. The CO capacity, in ml./100 ml., was assumed to be 1.34 times the hemoglobin, in Gm./100 ml.

The relationship of alveolar pCO to COHb saturation at toxic levels was evaluated after administration of CO by open-circut breathing from a tank containing approximately 0.3 per cent CO and 20 per cent O_2 in nitrogen. Blood and gas samples were collected simultaneously 5 to 15 minutes after breathing this mixture, and at intervals during the ensuing 1 to 4 hours.

When tests were made in rapid succession for comparison of different breathholding methods, 2 to 4 minutes were allowed between tests to permit return to a steady state.

RESULTS

The first object was to select an alveolar sampling maneuver which could be easily performed and at the same time yield maximal concentrations of CO in a highly reproducible fashion. It was hoped that such maximal values would approximate the hypothesized mean partial pressure of CO in the pulmonary capillary blood.

---

*Model 16 Liston-Becker Infrared CO_2 Analyzer, Spinco Division, Beckman Instruments, Inc., Springdale, Conn.

†Model 15 Liston-Becker Infrared CO Analyzer, fitted with 10¼ in. absorption cell, 5 in. filter cell filled with dry CO_2, 15¼ in. dummy cell and sapphire windows; Spinco Division, Beckman Instruments, Inc., Springdale, Conn. The instrument was used with an external meter, model 273, 0.1 milliampere range, 0.25 per cent accuracy, 200 scale division. Weston Electrical Instrument Corp., Newark, N. J. A 500 m.f.d. condenser was shunted across the meter for damping.

‡Hopcalite, Active. The Mine Safety Appliances Co., Pittsburgh, Pa.

Deep inspiration causes dilution of alveolar gas, and either breathholding or rebreathing into a closed system is needed to re-establish alveolar-capillary equilibrium. Experiences of others with rebreathing suggested difficulties with reproducibility.[6] Continuous end-tidal sampling was rejected because it requires steady state conditions and relatively complex apparatus. Accordingly, our studies concentrated on a breathing method.

A. Technique of Breathholding.—First, the significance of two variables had to be established; namely, the type and the duration of breathholding.

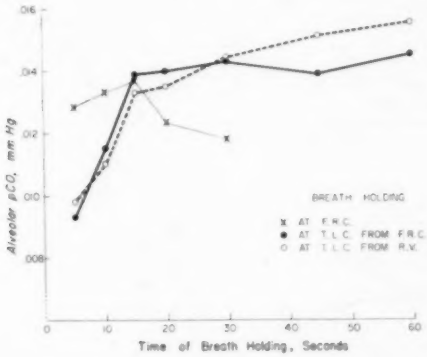


Fig. 1.—The effect of duration and type of breathholding on the partial pressure of CO in the alveolar gas (pCO).

Type of breathholding: This was investigated after 3 different maneuvers: (1) at the end of quiet expiration (at functional residual capacity); (2) after maximal inspiration from functional residual capacity; and (3) after maximal inspiration from residual volume. The first maneuver, breathholding at functional residual capacity, was most difficult to perform and maximal alveolar CO concentrations were obtained only transiently (Fig. 1). With the other 2 techniques, CO concentrations were approximately the same and the maneuvers were both easily performed. After some experimentation the third procedure, breathholding after full inspiration from residual volume, was chosen because it was felt that possibly a higher alveolar oxygen tension would be maintained for a longer period and, therefore, the onset of hypoxia delayed.[7]

Length of breathholding: The second variable to be studied was the length of breathholding and its relationship to alveolar pCO. As was expected, the CO concentration first rose with increasing length of breathholding, but then it fell progressively as apnea continued (Figs. 2 and 3). At low COHb levels the concentration rose only slightly and a plateau was maintained for almost one minute. At high levels the rise was steeper and maximal values were obtained at the end of *20 seconds* (Figs. 2 and 3). This time interval was chosen both as the most convenient and most productive period of breathholding.

B. Relationship Between Measured Alveolar pCO and COHb Saturation.—The alveolar pCO and COHb saturations were obtained simultaneously on 58

occasions in 9 subjects. Fig. 4 shows the relationships of these two values at 10-, 15-, 20-, and 30-second breathholding periods. The correlation was excellent but slightly alinear; a given change in COHb produced progressively greater changes in pCO as COHb saturation increased. Estimated mean curves were drawn and, as anticipated from the pCO versus breathholding relationships (Fig. 2), the 20- and 30-second curves superimposed (Fig. 5).

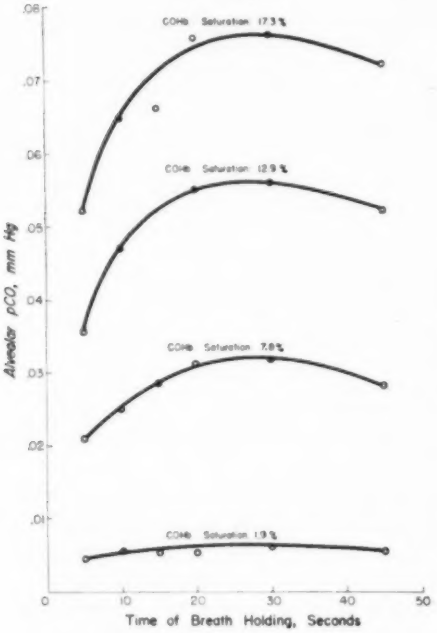

COHb Saturation 17.3 %
COHb Saturation 12.9 %
COHb Saturation 7.8 %
COHb Saturation 1.9 %
Alveolar pCO, mm Hg
Time of Breath Holding, Seconds

Fig. 2.

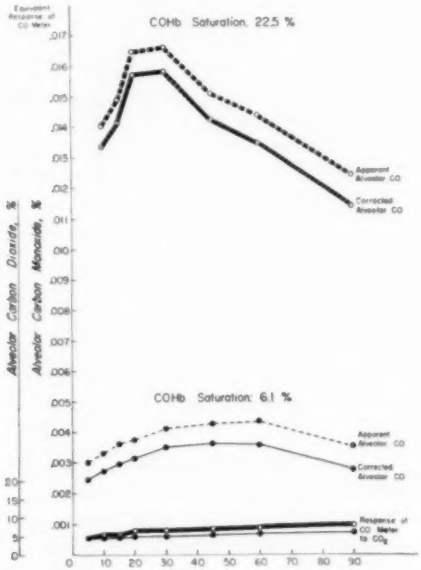


Fig. 3.

Fig. 2.—Variations of alveolar pCO with time of breathholding and with degree of COHb saturation.

Fig. 3.—Changes in alveolar CO concentration with time of breathholding in seconds and at low and high blood COHb saturation, with and without correction for the effect of CO_2 in the gas sample. The equivalent response of the CO meter to CO_2 is indicated.

The data from the 20-second breathholding maneuver were analyzed to ascertain the spread from the mean curve. The results were expressed in two ways: (1) as absolute deviation from the mean—90 per cent of the points fell within ± 1.3 per cent COHb saturation of this mean curve, and the average spread was ± 0.64 per cent saturation; and (2) as percentage deviation—90 per cent of the values fell within ± 10 per cent of the mean curve. Analyses of the 10-, 15-, and 30-second curves showed similar distribution of the points around the mean. Plotting of COHb per cent saturation against uncorrected CO meter reading resulted in a scatter which was slightly but significantly smaller, the increase in accuracy being about 5 per cent ($X^2 = 37.662$, $p < 0.001$).

C. Reproducibility.—The reproducibility of the method was ascertained in the following manner: serial 20-second alveolar samples were obtained both at

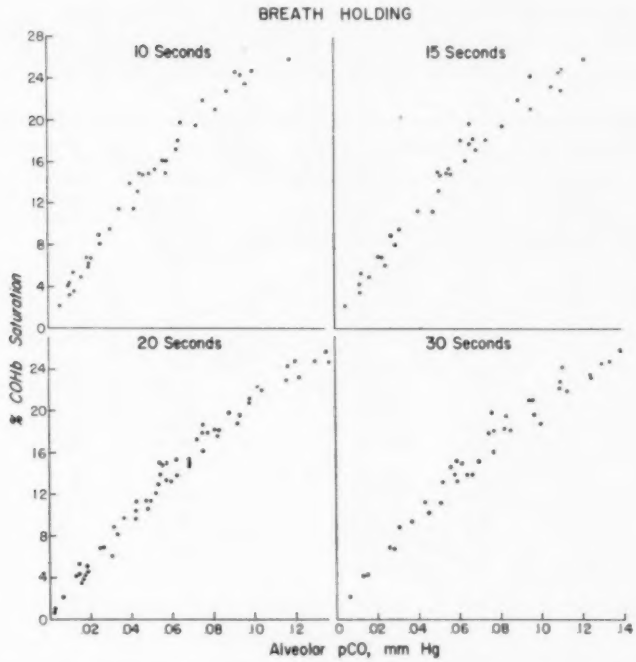


Fig. 4.—The relationship between simultaneously tested alveolar pCO and blood COHb saturation at 10, 15, and 30 seconds' breathholding. Nine normal subjects were examined on 58 occasions.

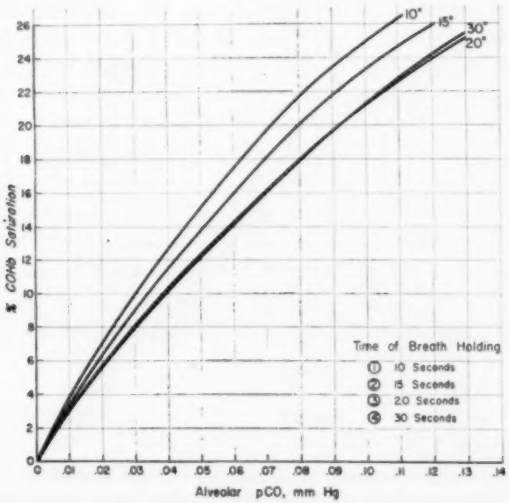


Fig. 5.—The relationship between alveolar pCO and blood COHb saturation: mean curves derived from the scattergrams of Fig. 4.

low and high COHb saturations (Table I). The analyses were reproducible to within ± 3.8 per cent of a given mean CO meter reading and within ± 4.7 per cent of a given mean pCO. It was thought that at high COHb levels there was

TABLE I. SERIAL ALVEOLAR 20-SECOND CO SAMPLES AT LOW AND HIGH COHb SATURATIONS

LOW SATURATION				HIGH SATURATION			
SUBJECT	UNCORR. METER READING	CORRECT* METER READING	ALVEOLAR pCO, mm. Hg	SUBJECT	UNCORR. METER READING	CORRECT METER READING	ALVEOLAR pCO, mm. Hg
M. E. S.	4.4	1.5	0.0026	M. E. S.	46.5	43.8	0.0735
	4.5	1.7	0.0030		48.3	45.7	0.0768
	4.3	1.6	0.0028		47.2	44.6	0.0749
	4.2	1.8	0.0032		48.2	45.6	0.0767
	4.8	2.4	0.0042		46.7	44.1	0.0740
	4.5	2.0	0.0035		46.3	43.7	0.0733
S. C.	4.0	1.2	0.0021	S. C.	67.5	64.7	0.1110
	4.6	1.7	0.0030		63.0	60.2	0.1029
	4.4	1.7	0.0030		63.3	60.5	0.1035
	4.8	2.1	0.0037		61.4	58.5	0.0998
	4.3	1.7	0.0030		61.6	58.7	0.1001
M. E. (1)	10.8	8.5	0.0150	M. E.	50.8	48.4	0.0804
	10.8	8.6	0.0151		46.5	44.1	0.0732
	10.7	8.6	0.0151		42.1	39.7	0.0656
	10.9	8.7	0.0154		44.9	42.7	0.0708
	11.0	8.7	0.0154		49.7	47.1	0.0785
	10.8	8.6	0.0151		48.4	45.8	0.0762
M. E. (2)	10.8	8.7	0.0154				
	11.2	9.1	0.0159				
	10.5	8.4	0.0148				
	10.8	8.6	0.0151				
	10.4	8.2	0.0144				
	10.9	8.7	0.0154				
R. H. J.	10.9	8.0	0.0138	R. H. J.	59.0	56.2	0.0959
	11.2	8.5	0.0145		60.4	57.7	0.0985
	11.2	8.4	0.0143		59.2	56.3	0.0961
	10.8	8.1	0.0140		57.6	54.8	0.0933
	10.7	8.2	0.0142		56.4	53.9	0.0919
	11.0	8.5	0.0145		56.6	53.9	0.0919
J. C. (1)	12.8	9.7	0.0169	J. C.	61.3	58.2	0.1005
	13.7	11.0	0.0191		59.8	56.9	0.0979
	13.0	10.1	0.0177		60.2	57.2	0.0987
	13.8	11.1	0.0193		58.9	55.6	0.0956
	13.8	10.8	0.0187		57.8	54.6	0.0939
	13.2	10.3	0.0180		57.8	54.6	0.0939
J. C. (2)	13.9	11.0	0.0191	E. A. G.	51.1	48.3	0.0822
	14.0	11.1	0.0193		49.1	46.3	0.0787
	13.5	10.6	0.0186		50.3	47.6	0.0810
	13.3	10.2	0.0179		50.2	47.4	0.0806
	13.0	10.1	0.0177		48.1	45.4	0.0772
	12.7	9.8	0.0172		48.8	46.2	0.0785
Mean (EX/N')	9.75		0.0125		54.00		0.0870
S.D.†	±0.309		±0.0006		±2.04		±0.0034
Coef. of variance	±3.17%		±4.65%		±3.77%		±3.89%

*Corrected for effect of CO_2.

†Standard deviation of the difference from individual means, where S.D. = $\frac{\sqrt{\text{E } (\overline{X} - X)^2}}{N' - N}$; $\overline{X}$ = mean of each subject's several values; X = individual value; N' = number of observation; and N = number of subjects.

sufficient elimination of CO from the blood during the period required for multiple testing to affect the reproducibility. Therefore, the accuracy of a single examination is probably better than that shown.

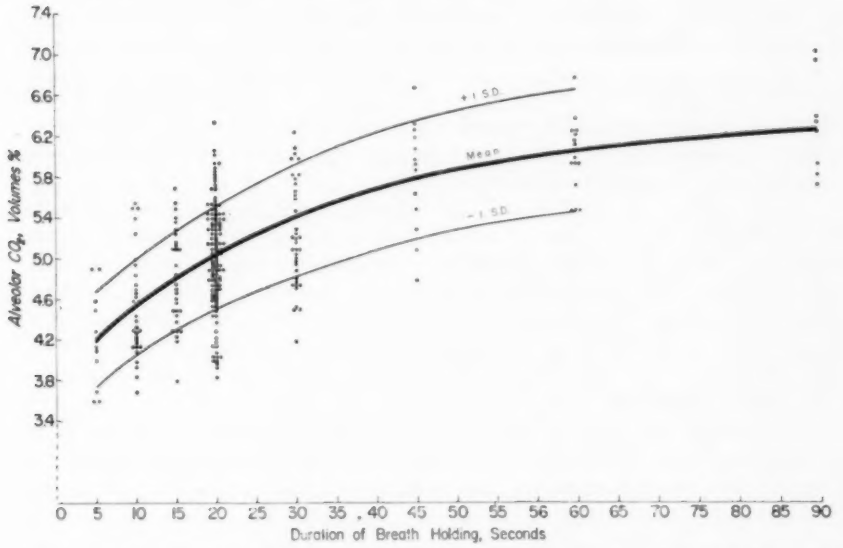


Fig. 6.—Alveolar carbon dioxide concentrations at various intervals of breathholding. Ten subjects were examined on 265 occasions.

D. Error Due to Presence of CO_2 in the Gas Sample.—Alveolar samples contained concentrations of CO_2 which, unlike CO, increased continuously with the length of breathholding (Fig. 6). At the very high gains employed, the CO meter had a measurable response to these CO_2 levels[3] so that the observed CO reading was slightly too high (Fig. 3). In this study appropriate corrections were made from the actually measured CO_2 content.

A diagram was constructed to eliminate the need for separate CO_2 analysis. Fig. 6 shows the measured alveolar CO_2 concentrations of 10 normal subjects observed on 265 occasions; both the mean and one standard deviation are indicated for breathholding periods from 5 through 60 seconds. For the 20-second interval the alveolar CO_2 concentration obtained on 132 occasions, averaged 5.03 ± 0.51 volumes per cent. Use of this diagram, instead of CO_2 analyses, should result in a maximal error in pCO of only ± 0.004 mm. Hg in normal subjects.

DISCUSSION

During breathholding there is an exchange of diffusible gases from areas of high pressure to those of low pressure: alveolar pCO should therefore rise asymptotically and the pO_2 should fall continuously. In fact, it is known that with our type of breathholding alveolar pO_2 decreases from initial abnormally

high values to below resting levels after 40 to 45 seconds.[7] Concerning pCO, however, our graphs show that it rises at first and then after 30 to 45 seconds, instead of reaching a plateau, falls progressively (Figs. 2 and 3).

The decline of pCO during the later period may be explained in part by the interrelation between the pressures of this gas and that of oxygen upon hemoglobin as defined by Haldane's first law. The observed drop in pCO is caused by the progressive decline of pO_2, if $\frac{M\ pCO}{pO_2} = \frac{COHb}{O_2Hb}$ and if the COHb saturation remains essentially unchanged during breathholding.[8] The decline of pCO is aggravated by yet another factor: the decay of COHb saturation during the time required for gathering of data.

The described fall in alveolar pCO required a definite choice of the duration of breathholding so that a portion of the curve could be examined before appreciable decay of oxyhemoglobin or carboxyhemoglobin had occurred. If maximal levels of alveolar pCO could be attained within a given breathholding period at all levels of COHb saturation, then the relation of a given maximal alveolar pCO to its COHb saturation should be relatively constant and predictable. This should provide an easy and precise method for indirect estimation of the COHb saturation.

Our studies have shown that essentially maximal levels of alveolar pCO are attained within 20 seconds of breathholding; that there is good reproducibility of the 20-second maneuver; and that the relation of alveolar pCO to the corresponding blood COHb saturation is remarkably similar for all subjects tested.

It is of interest to compare the 20-second carboxyhemoglobin dissociation curve of Fig. 5 with derived dissociation curves for blood with various oxygen saturations. If complete equilibration of CO between blood and alveoli occurred in 20 seconds then the observed effective pCO should fall somewhere between that of arterial and of venous blood. In Fig. 7 theoretical CO-Hb dissociation curves were constructed for venous blood (pO_2 = 35 mm. at 0 per cent COHb; pO_2 = 21.6 mm. at 25 per cent COHb),[8] mean pulmonary capillary blood (pO_2 = 85 mm.),[9] and arterial blood (pO_2 = 100 mm.),[9] using M = 245.[10] The observed 20-second curve practically superimposes upon that derived for the mean pulmonary capillary blood. With other values for M, perfect superimposition would not occur. The resemblance of our curve to the true dissociation curve cannot be definitely ascertained until more information is available concerning the value of M in the lowest ranges of COHb saturation.

The data shown were obtained at sea level with an average barometric pressure of 761 mm. Hg, and variations between 746 and 773 mm. Hg. These small changes should cause variations around the mean pCO of less than ± 3.0 per cent which is within the error of the method. However, at higher altitudes the pCO decreases significantly for a given concentration of CO. For example: at sea level a CO concentration of 0.012 per cent corresponds to an alveolar pCO of 0.0856 mm. Hg, compared with only 0.0556 mm. at 10,000 feet. If the demonstrated relationship of COHb saturation versus pCO (Fig. 5) remained constant at all altitudes, the illustrated drop in pCO should be associated with a drop

in COHb from 19 to 13 per cent (Fig. 5). However, Haldane's law shows that, because of the simultaneous decrease of pO_2, the relationship is changed. Actually, at any given altitude, the amount of COHb present can be predicted from the equation: $\text{COHb} = \frac{M\ pCO}{pO_2 + M\ pCO} \times$ total saturation.[11] From this, the COHb saturation for a given CO concentration can be calculated to be approximately the same at 10,000 feet as at sea level. This can be seen graphically in Fig. 7, realizing that the mean pulmonary capillary pO_2 at 10,000 feet would be much nearer the curve marked "venous blood." Thus, it would appear that a better estimate of the blood COHb at altitudes might be obtained by reference to a curve correlating COHb saturation with CO *concentration* (i.e., meter reading) rather than with pCO.

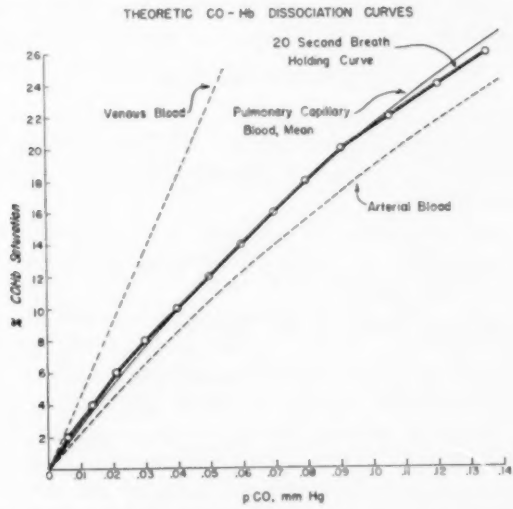


Fig. 7.—Comparison of the experimental 20-second carboxyhemoglobin "dissociation curve" (from Fig. 5) to theoretical curves constructed from Haldane's equation and assuming a pO_2 variously, of venous, mean pulmonary capillary, and arterial blood and M = 245.

Several investigators over the past 40 years have used equilibration techniques in man to investigate the relationship between COHb saturation and alveolar pCO.[4, 6, 10, 12-14] Henderson and associates[4] and Siösteen and Sjöstrand[12] gathered alveolar samples by rebreathing into a bag. Although the mean curves constructed from their data are comparable to our 20-second breathholding curve, only 30 to 60 per cent of their points fell within 10 per cent of the constructed curve. This unfavorable comparison must be attributed to better reproducibility of the breathholding maneuver over the rebreathing technique, as well as to the greater accuracy of our technical CO analysis of both blood and gas. Killick,[14] before reaching CO "acclimatization," obtained values derived from chamber air analysis which also fall at the upper portion of our 20-second curve.

The COHb-pCO relation at the 20-second breathholding period derives its stability from the fact that there are minimal changes in alveolar pCO in this portion of the breathholding curve (Figs. 2 and 3). A slight error in the timing maneuver causes little error in the estimate of blood COHb. At the end of much shorter breathholding periods, the alveolar pCO rises sharply, especially at high blood COHb levels, and a small timing error would lead to an appreciable error of COHb estimate. The choice of a 20-second period also is satisfactory from the clinical standpoint: in a study of over 100 untrained normal subjects there was only one individual who failed to perform the maneuver satisfactorily.[15] The variations which did occur between alveolar and blood CO values are felt to be the result mainly of experimental error in the determination of the CO capacity. However, neither biologic differences in the affinity of hemoglobin for CO nor uneven ventilation could be ruled out as a source of some variation.

SUMMARY AND CONCLUSIONS

In industrial or experimental situations where CO inhalation is a hazard, automatic alarming devices often cannot be used; calculation of degree of exposure is unreliable; and direct estimation of blood carboxyhemoglobin saturation is impracticable.

This study was undertaken to ascertain the relationship between blood carboxyhemoglobin (COHb) saturation and alveolar partial pressure of carbon monoxide (pCO) during breathholding.

During voluntary apnea after full inspiration, the alveolar pCO first rose abruptly, reached a short plateau between 20 and 30 seconds, and then declined. With breathholding at functional residual capacity the peak was more transient.

Simultaneous blood COHb (Range = 1.04 to 26.8 per cent saturation) and pCO analyses in 9 normal subjects on 58 occasions showed a slightly alinear but very close relationship: for 20 seconds' breathholding, 90 per cent of the points fell within ± 1.3 per cent COHb saturation of the mean curve and the average spread was ± 0.64 per cent saturation; or, 90 per cent of the points fell within ± 10 per cent of the mean curve. Correlation between COHb saturation and CO meter reading was even closer. Thirteen subjects performed 6 consecutive breathholding maneuvers in succession at both low and high COHb saturations. Analyses were reproducible within ± 3.8 per cent of a given mean CO meter reading and within ± 4.7 per cent of a given mean pCO.

The presence of CO_2 in the sample caused a measurable error; therefore, alveolar CO_2 concentrations were measured during breathholding in 10 subjects on 265 occasions. The CO_2 content after 20 seconds' breathholding averaged 5.03 ± 0.51 volumes per cent. Use of this mean value for CO meter correction, instead of separate CO_2 analysis, caused a maximal error in pCO of only ± 0.004 mm. Hg.

The curves from our data relating COHb saturation and alveolar pCO were compared to theoretical CO-Hb dissociation curves constructed according to Haldane's first law, assuming M = 245 and, variously, venous, mean pulmonary

capillary, and arterial oxygen saturations. Our 20-second curve superimposed almost exactly upon the theoretical curve plotted for a pO_2 of 85 mm. Hg or "mean pulmonary capillary" O_2 tension.

All studies were performed at sea level. From theoretical considerations it would appear that at higher altitude the demonstrated relationship between COHb saturation and alveolar pCO should not hold whereas that between COHb saturation and CO concentration should be almost the same as at sea level.

From these observations a simple method was devised for screening examination for COHb saturation of exposed personnel. A deepest breath is taken after maximal expiration, held for 20 seconds, and exhaled through a three-way valve. The last portion of the expired gas is collected in a rubber bag and analyzed for CO content with an infrared meter previously standardized with CO free air and 0.025 per cent CO from a tank. The reading is converted to pCO after correction for CO_2 in the sample. The COHb saturation is read from our CO-Hb dissociation curve either by reference to alveolar pCO or CO meter reading.

We wish to express our gratitude to Misses Mary Elizabeth Stone and Suzanne R. Crane for their technical assistance; we wish to thank Mr. Max Liston for directing our attention to the need of industry and the Armed Forces for a simple determination for COHb saturation.

REFERENCES

1. Forbes, W. H., Sargent, F., and Roughton, F. J. W.: The Rate of Carbon Monoxide Uptake by Normal Man, Am. J. Physiol. **143**: 594, 1945.
2. Consolazio, W. V., White, W. A., Jr., and Behnke, A. R.: Formulation of the Principal Factors Affecting the Rate of Uptake of Carbon Monoxide by Man, Am. J. Physiol. **147**: 352, 1946.
3. Gaensler, E. A., Cadigan, J. B., Ellicott, M. F., Jones, R. H., and Marks, A.: A New Method for Rapid Precise Determination of Carbon Monoxide in Blood, J. Lab. & Clin. Med. **49**: 945, 1957.
4. Henderson, Y., Haggard, H. W., Teague, M. C., Prince, A. L., and Wunderlich, R. M.: Physiological Effects of Automobile Exhaust Gas and Standards of Ventilation for Brief Exposures, J. Indust. Hyg. **3**: 79, 1921.
5. Burwell, C. S., and Robinson, G. C.: A Method for the Determination of the Amount of Oxygen and Carbon Dioxide in the Mixed Venous Blood of Man, J. Clin. Invest. **1**: 47, 1924.
6. Sjöstrand, T.: A Method for the Determination of Carboxyhemoglobin Concentrations by Analysis of Alveolar Air, Acta physiol. scandinav. **16**: 201, 1948.
7. Ferris, E. B., Engel, G. S., Stevens, C. D., and Webb, J.: Voluntary Breathholding. III. The Relation of the Maximum Time of Breathholding to the Oxygen and Carbon Dioxide Tensions of Arterial Blood, With a Note on Its Clinical and Physiological Significance, J. Clin. Invest. **25**: 734, 1946.
8. Roughton, F. J. W., and Darling, R. C.: The Effect of Carbon Monoxide on the Oxyhemoglobin Dissociation Curve, Am. J. Physiol. **141**: 17, 1944.
9. Lilienthal, J. L., Jr., Riley, R. L., Proemmel, D. D., and Franke, R. E.: Experimental Analysis in Man of Oxygen Pressure Gradient From Alveolar Air to Arterial Blood During Rest and Exercise at Sea Level and at Altitude, Am. J. Physiol. **147**: 199, 1946.
10. Carlsten, A., Holmgren, A., Linroth, K., Sjöstrand, T., and Ström, G.: The Relationship Between Low Values of Alveolar Carbon Monoxide Concentrations and Carboxyhemoglobin Percentage in Human Blood, Acta physiol. scandinav. **31**: 62, 1954.
11. Lilienthal, J. L., Jr., Riley, R. L., Proemmel, D. D., and Franke, R. E.: The Relationships Between Carbon Monoxide, Oxygen, and Hemoglobin in the Blood of Man at Altitude, Am. J. Physiol. **145**: 351, 1946.
12. Siösteen, S. M., and Sjöstrand, T.: A Method for the Determination of Low Concentrations of Carbon Monoxide in the Blood and the Relation Between the CO

Concentration in the Blood and That in the Alveolar Air, Acta physiol. scandinav. **22**: 129, 1951.

13. Dahlström, H.: A Critical Study of Sjöstrand's Method for Determination of Carboxyhemoglobin and the Total Hemoglobin Content of the Human Body, Acta physiol. scandinav. **33**: 296, 1955.
14. Killick, E. M.: The Nature of the Acclimatization Occurring During Repeated Exposure of the Human Subject to Atmospheres Containing Low Concentrations of Carbon Monoxide, J. Physiol. **107**: 27, 1948.
15. Cadigan, J. B., Ellicott, M. F., Jones, R. H., Kanagami, H., and Gaensler, E. A.: The Normal Single-breath Carbon Monoxide Diffusing Capacity of the Lungs and Its Relationship to Age, Sex, Stature, and Lung Volumes. (To be published.)

THE DIFFERENTIAL DIAGNOSIS OF BILIARY TRACT OBSTRUCTION WITH RADIOACTIVE ROSE BENGAL

R. A. Nordyke, M.D., and W. H. Blahd, M.D.
Los Angeles, Calif.

With the Technical Assistance of Lucille Shoop, R.N., Billie Jean Kinney, and Marianne Lederer

A NEW approach to the differential diagnosis of intra- and extrahepatic obstruction has been made possible by the recent development of I^{131}-labeled rose bengal. After intravenous injection the dye is cleared from the blood almost entirely by the polygonal cells of the liver[1, 2] and is subsequently excreted into the biliary system and duodenum. Serial external scintillation counting at the head, liver, and lower abdomen allows the detection of changing concentrations of dye radioactivity: counting over the stable vascular supply of the head reflects the levels of dye in the blood[3]; counting over the liver reflects the uptake of dye by the liver cells[4-10]; and counting over the abdomen reflects the changing concentrations of dye in both the abdominal blood and the intestinal lumen. By means of appropriate graphing techniques the component of the abdominal curve contributed by blood can be separated from the component due to dye within the intestine. It therefore becomes possible to obtain the rate of disappearance of rose bengal from the blood, its uptake by the liver, its time of arrival in the duodenum and its accumulation in the intestine. These data yield useful criteria for distinguishing between intra- and extrahepatic obstruction of the biliary tract.

METHODS

Patients (nonfasting) were placed in a comfortable semireclining position. Three wide-angle scintillation detector tubes were used in most of the studies and positioned as follows: (1) horizontally against the head, centered at the right ear; (2) anteriorly over the liver; and (3) over the left lower abdominal quadrant (LLQ), directed slightly inferiorly (Fig. 1). After a background count in each position, 10 to 25 μc I^{131} rose bengal in 2 to 5 ml. of normal saline was injected intravenously such that the 5 minute counting rate at the head was about 10 times background. Counts were then obtained every 2 minutes for 30 minutes and every 5 minutes for ½ to 2 hours thereafter. In 2 patients who had indwelling T tubes in the common bile duct, timed samples of bile were taken simultaneously for deep-well scintillation counting. In a third patient the dye was injected into the T tube before the procedure of external counting. Another patient was given 25 μc I^{131} rose bengal orally.

In order to compare the change of dye concentration at different body sites and to compare one patient with another, a semilog plot was used, with counts per second on the

From the Radioisotope Service, Wadsworth General Hospital, Veterans Administration Center, Los Angeles, and the Department of Medicine, University of California at Los Angeles Medical Center.

Received for publication Sept. 23, 1957.

ordinate and time on the abscissa. Net counts (gross minus background) at each position were plotted and the best line drawn through the obtained data (Fig. 2, *A*). On the assumption that most dye within the abdomen is vascular in the early phase, each curve was moved, by simple parallel geometric displacement, to start at 15 minutes and at 100 counts per second (Fig. 2, *B*). A curve representing the difference between the displaced head and LLQ curves was then plotted, from which the time of arrival of dye and its rate of accumulation in the intestine could be ascertained.

Fig. 1.—Positioning of scintillation counters at head, liver, and left lower abdominal quadrant.

RESULTS

A. *Studies in the Dynamics of Injected Rose Bengal.*—

1. *External head counts:* After the intravenous injection of radioactive rose bengal serial scintillation counting rates at the head approximate the blood disappearance of the dye (Fig. 3). The curve obtained in this manner declines somewhat more slowly than the curve described by serial blood samples which have been counted in a deep-well counter. This difference is about 15 per cent at one hour, most of which occurs after the head counting rate drops below 4 times background and it probably represents some scattered radiation from other areas, mainly liver, gall bladder, and intestine, along with some tissue binding. The head was chosen since it has a large and stable vascular supply located at a distance from the accumulations of dye in the abdomen and provides excellent testing reproducibility.

2. *External left lower abdominal quadrant counts:* In order to separate abdominal radioactivity into its component parts (blood and intestinal radioactivity) 5 studies were done as follows:

(*a*) Twenty-five microcuries I^{131} rose bengal was placed in a soluble capsule and given orally to a normal patient, after which external counting was done

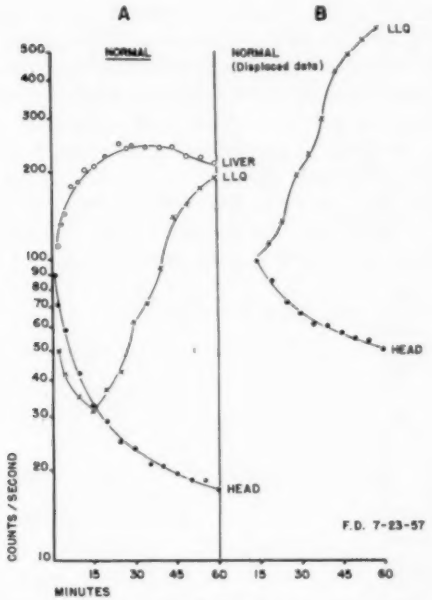


Fig. 2.—*A*, Typical normal curves described by semilog plots of the counting rates obtained at the head, liver and lower abdomen following the injection of a tracer dose of radioactive rose bengal. *B*, The same head and LLQ curves displaced such that both have the same point of origin, i.e., 15 minutes and 100 c/s, to permit the direct comparison of rates of change of dye radioactivity.

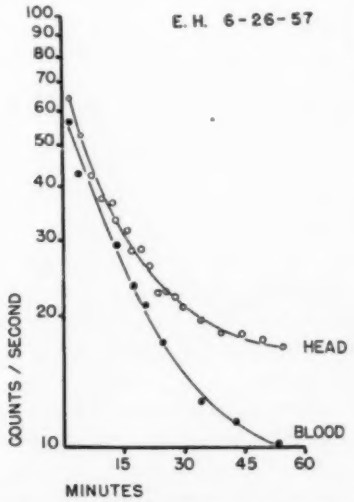


Fig. 3.—Serial counting rates of 0.5 ml. blood samples and external counting rates at the head, obtained simultaneously.

in the usual manner (Fig. 4). There was essentially no change in the liver or head counting rates during this experiment. The lower abdominal counter detected little until about 30 minutes, after which it rose in an undulating fashion. Both the head and the liver detectors recorded counting rates higher than background, representing scattered radiation from the dye passing through the intestinal tract, the liver count being higher because of proximity to the dye. Both, however, remained essentially constant throughout the 2 hours, demonstrating that there was no appreciable enterohepatic recirculation. The LLQ rise occurred as the dye presented itself beneath the abdominal scintillation counter and the height of the curve indicated the amount of dye present.

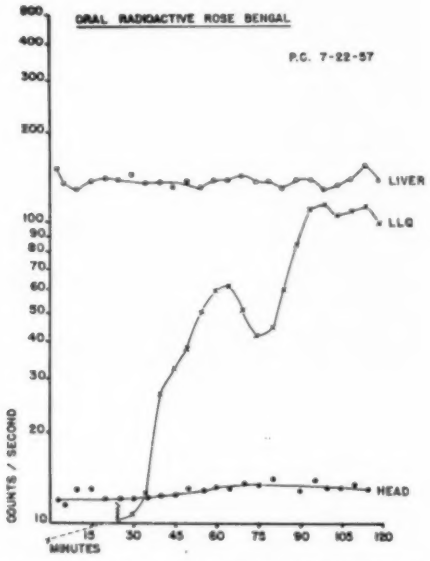


Fig. 4.—Serial external counting rates at head, liver, and LLQ following the oral ingestion of 25 μc I^{131} rose bengal.

(*b*) Fifteen microcuries I^{131} rose bengal was injected directly into the T tube and common bile duct of a postcholecystectomy patient. The head count rose immediately to twice background and remained essentially constant through the 2-hour test period. The liver count was 6 times background at 2 minutes and decreased slightly thereafter. The LLQ counts increased to 10 times background within 1 minute and remained steady, except for minor fluctuations, throughout the period of study.

(*c*) Fig. 5 presents data on a patient who had had a gastric carcinoma with jaundice. At operation, 21 days before this study, the common bile duct was found to be occluded by a large mass. A cholecystotomy was done and the bile flow was exteriorized; the jaundice subsided and the stools remained clay-colored. After the intravenous injection of rose bengal, external counting was

done, and the concentration of radioactivity in 0.5 ml. aliquots of bile was determined. The LLQ and head curves approximately paralleled each other for the entire 2-hour study period. The dye appeared in the bile at about 12 minutes and the concentration increased rapidly thereafter.

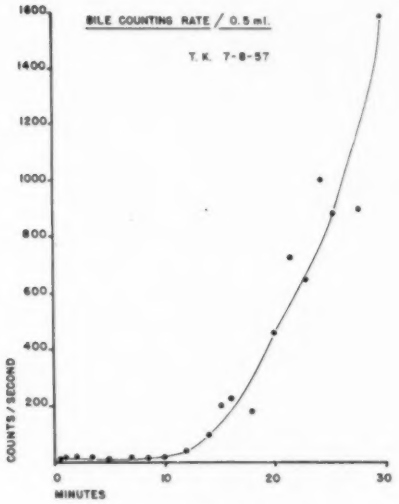


Fig. 5.

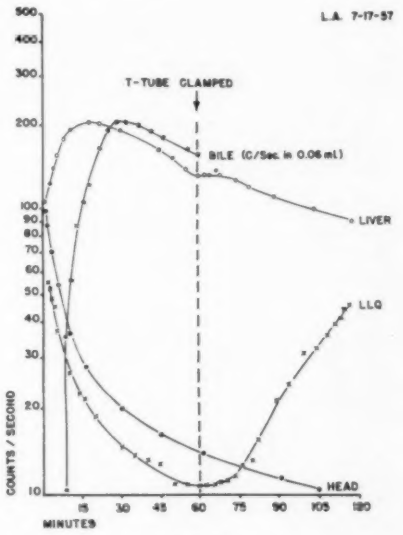


Fig. 6.

Fig. 5.—Plot of the radioactivity of bile aliquots obtained from cholecystotomy tube in patient with complete block of common bile duct due to carcinoma, after intravenous injection of 25 μc I^{131} rose bengal.

Fig. 6.—External scintillation counting and bile counting rates in patient with T tube in common bile duct and stones present in the distal bile duct. T tube was clamped at one hour.

(*d*) Fig. 6 represents a patient who had a cholecystectomy with removal of a 1 by 1½ cm. stone from the common bile duct. This study was done 15 days after surgery and 3 hours after cholangiography through the T tube had demonstrated 2 stones remaining in the duct distally. Clear, olive-colored bile flowed freely externally. For the first hour the head counting rates decreased rapidly, paralleled by the rates at the LLQ. The liver curve rose and later declined, in normal fashion. Dye appeared in the bile in quantity in the 8 to 10 minute aliquot, and the curve of its concentration paralleled both the rise and the fall of the liver curve, but was delayed in time. At one hour the external T tube arm was clamped off, after which there was a slight flattening of the liver curve and a continued decline of the head curve. There was an abrupt increase in the LLQ curve, but the rate of accumulation was slower than normal.

(*e*) This patient had a cholecystectomy 26 days prior to this study, at which time several small stones were removed from a moderately dilated common bile duct. Most of the bile was flowing into the intestine, and only intermittent flow appeared in the exterior arm of a common duct T tube. For 16 minutes

TABLE I. CHARACTERISTICS OF CURVES OBTAINED BY SERIAL EXTERNAL SCINTILLATION COUNTING AT THE HEAD, LIVER, AND LEFT LOWER ABDOMINAL QUADRANT (LLQ) AFTER THE INTRAVENOUS INJECTION OF 25 μc I^{131} ROSE BENGAL

PATIENT	DATE	DIAGNOSIS	LIVER 10 TO 2 MIN. RATIO	HEAD 2 TO 10 MIN. RATIO	HEAD 20 TO 5 MIN. RATIO	INTESTINAL ARRIVAL TIME (MINUTES)	LLQ ADJUSTED NET COUNTING RATE AT 1 HR.	COMMENT
				Normals				
W. R.	8/ 7/57	Normal	1.84	1.82	0.45	14	650	
G. M.	7/12/57	Normal	1.82	1.70	0.49	15	370	
F. D.	7/23/57	Normal	1.60	1.57	0.50	14	600	
D. J.	9/ 4/57	Normal	1.83	1.77	0.48	14	270	
I. A.	9/ 5/57	Normal	1.60	1.88	0.47	14	500	
O. D.	9/ 5/57	Normal	1.66	1.86	0.44	10	355	
P. Q.	9/ 5/57	Normal	1.49	2.20	0.45	5	225	
				Extrahepatic Obstruction				
L. A. (1)	6/18/57	CBD stone	2.25	1.79	0.48	30	41	1 by 1½ cm. stone in CBD near cystic duct
L. A. (2)	7/19/57	Postop., stones in distal CBD	1.56	1.89	0.46	*	0	T tube in CBD. Stones in CBD distal to T tube. External bile flow (Fig. 6)
H. F. (1)	6/12/57	CBD stones	†	†	0.64	45	20	Small stones in moderately dilated CBD
H. F. (2)	7/ 8/57	13 days postop.	†	1.66	0.49	18	115	T tube in CBD. Intermittent external bile flow
E. S.	7/24/57	Ca. head pancreas, 14 days postop.	1.52	1.47	0.60	27	80	Surgery done 2 weeks after onset of jaundice; at time of study, jaundice not clearing well
H. M.	8/ 2/57	Lymphosarcoma with abdominal mass	1.57	1.56	0.77	30	10	Jaundice 2 weeks' duration
T. K.	7/ 8/57	Ca. stomach with extension to CBD, 21 days postop.	1.28	1.37	0.68	*	10	Choledochostomy with bile appearing externally. Clay stools. Jaundiced for 6 weeks prior to surgery (See Fig. 5)
F. H. (1)	5/ 8/57	Ca. head pancreas	†	†	0.70	60	0	Jaundice 3 weeks before study
F. H. (2)	6/13/57	Postop. 23 days	†	†	0.75	30	19	Doing poorly postoperatively
G. H. (1)	8/ 9/57	Ca. head pancreas	1.20	1.20	0.77	*	0	Jaundice 7 weeks before study
G. H. (2)	8/29/57	Postop. 14 days	1.55	1.42	0.64	47	17	Sharp increase in intestinal counting rate after 47 min., to 355 at 105 min.

G. H. (3)	9/ 5/57	Postop. 21 days	1.59	1.47	0.62	12	160	Doing well
W. D. (1)	6/ 6/57	Ca. head pancreas preop.	†	†	0.77	*	13	Jaundice 5 weeks. Occasional light brown stools. SB varying between 10-20 mg. per cent
W. D. (2)	6/21/57	Preop.	1.27	1.43	0.71	55	7	
W. D. (3)	6/26/57	Preop.	1.27	†	0.72	*	7	
W. D. (4)	7/19/57	Postop. 11 days	1.56	1.52	0.54	28	195	Cholecystojejunostomy on 7-8-57. Uneventful recovery. SB 3.5 mg. per cent
W. D. (5)	8/ 8/57	Postop. 30 days	1.42	1.41	0.61	50	12	Chills and fever; jaundice still present. Stools brown
				Intrahepatic Obstruction				
S. H.	7/15/57	Well-compensated Laennec's cirrhosis	1.73	1.77	0.52	10	156	
W. W.	6/28/57	Viral hepatitis, convalescent	1.22	2.0	0.43	8	385	No residual jaundice; ambulatory
R. S.	7/10/57	Moderately decompensated L. cirrhosis	1.39	1.31	0.72	15	82	Hepatosplenomegaly; spider angiomata. SB 2.1 mg. per cent
E. R.	7/15/57	Moderately decompensated L. cirrhosis	1.46	1.64	0.60	12	32	Hepatosplenomegaly; spider angiomata. CF 2/3. TT 12 units. TF 2+. SP 2.6/4.5. SB 0.39 mg. per cent
D. K. (1)	6/14/57	Viral hepatitis, early stage	1.11	1.05	0.90	*	0	SB 24 mg. per cent. Jaundice 6 days' duration
D. K. (2)	6/20/57	Viral hepatitis	1.23	1.20	0.76	14	105	Much improved clinically; jaundice decreased
T. H. (1)	7/17/57	Viral hepatitis	1.06	1.13	0.94	10	28	SB 16.9 mg. per cent. CF 3/4+. TT 6.9 units. A. P'tase 7.2 units. SP 3.7/2.0
T. H. (2)	7/19/57	Viral hepatitis	1.12	1.25	0.82	*	32	SB 14.8 mg. per cent
T. H. (3)	7/23/57	Viral hepatitis	1.27	1.30	0.71	16	380	CF 4/4+. TT 8.3 units. TF 2+. A. P'tase 5.6 units. SGOT 320 units
T. H. (4)	7/26/57	Viral hepatitis	1.38	1.62	0.59	8	155	SB 4.15 mg. per cent. CF 3/4+. TT 10.0 units. TF 1+. A. P'tase 4.6 units
T. H. (5)	7/29/57	Viral hepatitis	1.54	1.49	0.57	8	265	CF 3/3+. TT 15.2 units. TF 2+. A. P'tase 4.9 units. SGOT 180 units. B.S.P. 14 per cent. SB 3.40 mg. per cent

TABLE I—CONT'D

PATIENT	DATE	DIAGNOSIS	LIVER 10 TO 2 MIN. RATIO	HEAD 2 TO 10 MIN. RATIO	HEAD 20 TO 5 MIN. RATIO	INTESTINAL ARRIVAL TIME (MINUTES)	LLQ ADJUSTED NET COUNTING RATE AT 1 HR.	COMMENT
T. H. (6)	8/ 2/57	Viral hepatitis	1.60	1.60	0.53	8	240	SB 3.0 mg. per cent. SGOT 63 units. CF 2/3. TT 13.6 units. TF 1+. A. P'tase 4.2 units
T. H. (7)	8/ 7/57	Viral hepatitis	1.65	1.61	0.50	10	460	CF 2/3+. TT 13.6 units. TF 1+. SB 2.48 mg. per cent. B.S.P. 11 per cent
W. L.	7/18/57	Decompensated L. cirrhosis	1.11	1.05	0.95	16	30	Hepatomegaly with ascites. SB 5.6 mg. per cent. SP 2.2/5.5
E. S.	8/13/57	Decompensated L. cirrhosis	1.13	1.08	0.83	6	10	
C. L. (1)	6/13/57	Decompensated L. cirrhosis	1.12	1.04	0.87	*	0	Hepatosplenomegaly. Minimal ascites. CF 2/3+. TT 4.6 units. SB 3.6 mg. per cent
C. L. (2)	8/13/57	Decompensated L. cirrhosis	1.10	1.09	0.94	*	0	CF 2/4+. TT 6.0 units. SB 4.3 mg. per cent
J. A.	8/26/57	Decompensated L. cirrhosis	1.07	1.05	0.94	*	7	CF 3/4+. TT 11.6 units. SB 27.1 mg. per cent. SP 0.57/6.88. A. P'tase 8.6 units

Key to abbreviations: CBD = common bile duct; TT = thymol turbidity, units; TF = thymol flocculation, 1 to 4+; SB = serum bilirubin; A. P'tase = serum alkaline phosphatase, Shinowara units; CF = cephalin flocculation at (24/48) hours; B.S.P. = Bromsulphalein dye retention at 45 min. after intravenous injection of 5 mg./Kg. body weight; SGOT = serum glutamic oxaloacetic transaminase; SP = serum proteins (albumin/globulin), grams per cent.

*Data not obtainable.

†Data not obtained.

the LLQ curve paralleled the head curve, after which it diverged and climbed rapidly. The exteriorized bile concentration was first detected in traces in the 16-minute aliquot, and then increased rapidly. The time of arrival of the dye in the common bile duct and the time of divergency of the LLQ and head curves were essentially the same.

3. *External liver counts:* The plot of serial scintillation counting rates over the liver describes a curve which in normal patients rises rather rapidly for the first 15 to 30 minutes, the 10 to 2 minute counting ratio being over 1.49 in 7 normal patients (Table I). When plotted on semilog paper this rise is approximately the mirror image of the declining head counts, but tends to be slightly flatter in some cases because of early excretion away from the liver area. After about 30 minutes the curve declines slowly in normal individuals but remains elevated in obstruction from any cause.

B. *Clinical Results.—*

1. *Normals:* Fig. 2 illustrates typical results in a normal person. Serial counting rates at the head decrease rapidly at first and then more slowly. In a group of 41 normal fasting persons the 20 to 5 minute counting ratio ranged between 0.39 and 0.50, with a mean of 0.46.[3] The gross LLQ curve essentially parallels the head curve for about 15 minutes after which it diverges and rises rapidly. The difference between the displaced head and LLQ curves at one hour was over threefold in 7 normal persons. The liver curve rises for 15 to 30 minutes, remains stable for a short period and then gradually declines.

2. *"Complete" obstruction of the biliary tract:* This group is arbitrarily defined as those patients in whom the difference between the displaced head and LLQ curves was below 30 counts per second at one hour. In our series this included patients with common bile duct stones, extrinsic tumorous obstructions of the common bile duct, the early stages of viral hepatitis, and decompensated Laennec's cirrhosis.

3. *Comparison of curves in "complete" intra- and extrahepatic obstruction:* Table II lists all patients with biliary tract obstruction in whom the displaced LLQ-head difference at one hour is 30 counts per second or less. In these patients the intestinal arrival time was often difficult to determine precisely. The slopes of the liver and head curves were steeper in all cases of extrahepatic obstruction and could be roughly correlated with the length of time the jaundice was present. However, even after 7 weeks of jaundice due to extrinsic carcinoma the liver curve was not depressed to the level of the intrahepatic cases. The 10 to 2 minute counting ratios in 6 patients with intrahepatic obstruction of viral hepatitis and cirrhosis ranged from 1.06 to 1.13, while the ratios in all extrahepatic cases with severe obstruction ranged from 1.20 to 1.57. As recovery from the disease occurred, regardless of etiology, the curve became progressively steeper until it reached the normal range (see T. H., Table I).

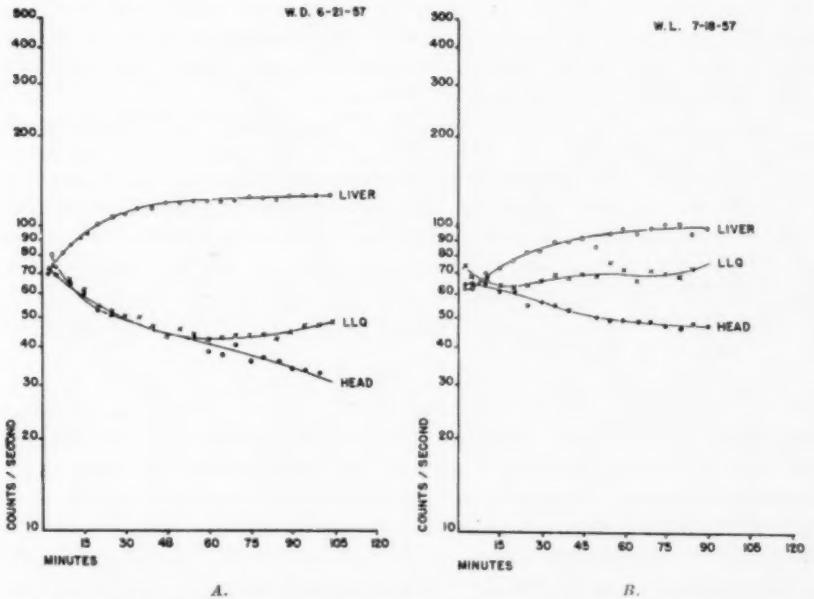
4. *"Partial" obstruction of the biliary tract:* This category defines those subjects in whom the difference between the displaced head and LLQ curves lies between about 30 and 100 counts per second at one hour.

TABLE II. CHARACTERISTICS OF CURVES IN SEVERE INTRA- AND EXTRAHEPATIC OBSTRUCTIONS OBTAINED BY SERIAL EXTERNAL SCINTILLATION COUNTING AT THE HEAD, LIVER, AND LEFT LOWER ABDOMINAL QUADRANT (LLQ) AFTER THE INTRAVENOUS INJECTION OF 25 μC I^{131} ROSE BENGAL

PATIENT	DATE	DIAGNOSIS	LIVER 10 TO 2 MIN. RATIO	HEAD 2 TO 10 MIN. RATIO	HEAD 20 TO 5 MIN. RATIO	INTESTINAL ARRIVAL TIME (MINUTES)	LLQ ADJUSTED NET COUNTING RATE AT 1 HR.
		Severe Intrahepatic Obstruction					
D. K. (1)	6/14/57	Viral hepatitis, early stage	1.11	1.05	0.90	*	0
T. H. (1)	7/17/57	Viral hepatitis, early stage	1.06	1.13	0.94	10	28
W. L.	7/18/57	Decompensated L. cirrhosis	1.11	1.05	0.95	16	30
E. S.	8/13/57	Decompensated L. cirrhosis	1.13	1.08	0.83	6	10
C. L. (1)	6/13/57	Decompensated L. cirrhosis	1.12	1.04	0.87	*	0
C. L. (2)	8/13/57	Decompensated L. cirrhosis	1.10	1.09	0.94	*	0
J. A.	8/26/57	Decompensated L. cirrhosis	1.07	1.05	0.94	*	7
		Range	1.06 to 1.13	1.04 to 1.13	0.83 to 0.95	<16	0 to 30
		Mean	1.10	1.07	0.91	*	10.7
		Severe Extrahepatic Obstruction					
H. F. (1)	6/12/57	CBD stones	†	†	0.64	45	20
H. M.	8/ 2/57	Lymphosarcoma with abdominal mass	1.57	1.56	0.77	30	10
F. H. (1)	5/ 8/57	Ca. head pancreas	†	†	0.70	60	0
G. H. (1)	8/ 9/57	Ca. head pancreas	1.20	1.20	0.77	*	0
W. D. (2)	6/21/57	Ca. head pancreas	1.27	1.43	0.71	55	7
		Range	1.20 to 1.57	1.20 to 1.56	0.64 to 0.77	30 to 60	0 to 20
		Mean	1.35	1.40	0.72	47	7

CBD = common bile duct.
*Data not obtainable.
†Date not obtained.

5. *Comparison of curves in "partial" intra- and extrahepatic obstruction:* Two rather distinct patterns were evident in these patients. Fig. 7, *A* demonstrates the results in a patient with obstruction of the common bile duct due to pancreatic cancer. Jaundice had been present for 5 weeks. It is notable that the rate of rise of the liver curve, though less than normal, is fairly rapid, the 10 to 2 minute counting ratio being 1.27, and the time of divergence of the head and LLQ curves is delayed to 55 minutes. The second pattern (Fig. 7, *B*) has occurred in our series consistently in the intrahepatic obstruction of viral hepatitis and decompensated Laennec's cirrhosis. The two curves diverge early; and the liver curve is markedly flattened, the 10 to 2 minute ratio in this case being 1.11. The intestinal arrival time in patients with partial intrahepatic obstruction, obtained by noting the point of divergence of the head and LLQ curves, ranged between 8 and 17 minutes, with a mean of 11.5 minutes. In partial extrahepatic obstruction the range was 30 to 60 minutes, with a mean of 42 minutes.



A. *B.*

Fig. 7.—Scintillation counting rates at head, liver, and LLQ in parital obstruction. *A*, Extrahepatic obstruction with jaundice of 5 weeks' duration, due to pancreatic carcinoma. *B*, Intrahepatic obstruction due to decompensated Laennec's cirrhosis.

6. *Activity in the thyroid gland:* External scintillation counting was done over the thyroid gland in 6 patients at the end of 2 and 24 hours, to determine the quantity of I^{131} present. The total gland activity was never higher than 0.6 μc in patients with a single test. In one patient in whom the test was repeated 7 times within 3 weeks (T. H.), using 25 μc I^{131} rose bengal for each test, the thyroid at no time contained over 2.0 μc.

DISCUSSION

The foregoing studies indicate that serial external scintillation counting at the head, liver, and abdomen after the intravenous injection of radioactive rose bengal will provide the rate of blood disappearance of the dye, the rate of its uptake by the liver, the time required to excrete dye into the duodenum, and the rate of accumulation of dye in the intestine. Each of these elements is useful in the differential diagnosis of intra- and extrahepatic biliary tract obstruction.

In partial obstruction the most important differential fact is the arrival time of the dye in the intestine, which is determined by the time of divergence of the head and LLQ curves. Caroli, Tanasoglu, and Cohen[11] and subsequently others[12, 13] observed that after the intravenous administration of Bromsulphalein dye in patients with extrahepatic obstruction the first appearance of the dye in the duodenum (determined by intubation) was delayed to about twice the normal time. In patients with intrahepatic obstruction, on the other hand, the arrival time was normal or decreased. Our studies with radioactive rose bengal have confirmed this. The reason for the phenomenon is not known, but an hypothesis might be advanced on the basis of the difference in liver pathology following the different types of obstruction.

Extrahepatic obstruction of the biliary tract causes diffuse morphologic changes of the polygonal cells within 24 to 48 hours.[14] Every cell appears to be about equally involved, as a result of increased pressure in the biliary system. Delayed dye excretion by the cells, with little impairment of dye uptake, is an early functional change; more prolonged extrahepatic obstruction delays the excretion time further and gradually reduces the uptake ability of the cells. Moreover, this delay in excretion remains for a variable period after the obstruction has been removed, suggesting that it is due to cellular injury (Fig. 8 and Table I, W. D., and G. H).

Intrahepatic obstruction, on the other hand, is spotty, whether it primarily affects the polygonal or the bile canalicular cells. Even in necropsy specimens of liver necrosis due to viral hepatitis[15] or in carbon tetrachloride poisoning of animals,[16] some areas contain normal and hyperplastic cells. It seems likely that both the uptake and excretion functions of the liver are carried on by a small percentage of cells in these areas; therefore, whatever quantity of dye appears in the intestine will appear at the normal time or earlier.

Most cases of jaundice can be classified as either intra- or extrahepatic on the basis of the intestinal arrival time of radioactive rose bengal alone. However, when the obstruction is nearly complete this method cannot be used. In such cases the early part of the liver uptake curve (or its mirror image, the head curve) must be examined. When there is severe primary damage to or malfunction of the polygonal cells, the slope of the liver uptake curve is depressed so that the 10 to 2 minute ratio is 1.13 or less (Table II). In complete extrahepatic obstruction the liver curve is progressively depressed, but in our series the 10 to 2 minute ratio was 1.20 or over even after 7 weeks of jaundice,

which is considerably above the range found in severe intrahepatic obstruction. The uptake function of the liver cells seems to be relatively insensitive to injury from increased biliary tract pressure in contrast to excretion function.

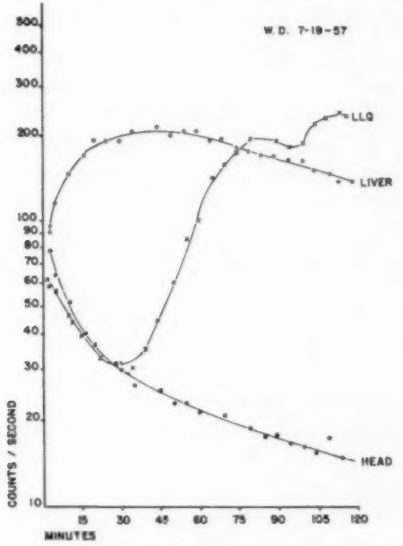


Fig. 8.

Chlorpromazine jaundice is a special problem in which the polygonal cells are relatively little injured and the bile canaliculi are plugged in a spotty fashion.[17] Therefore, it might be predicted on the basis of the above hypothesis that the liver uptake of dye would be normal or moderately decreased, the time of arrival of dye in the intestine would be normal, and the intestinal dye accumulation would be decreased. Such liver uptake curves have been reported in 7 cases by Brown and Glasser[8] and 1 by Wood and Korst,[10] but further data are lacking. Chlorpromazine jaundice with complete obstruction of dye from the intestine would theoretically be difficult to distinguish from complete extrahepatic obstruction of short duration, although serial studies may aid in the differential.

There are certain areas where further experience will be necessary before final evaluation can be made. One is the differential between complete obstruction due to severely decompensated cirrhosis or viral hepatitis and complete extrahepatic obstruction lasting over about 10 weeks. Another is in possible falsely delayed arrival time of the dye in the intestine. Both the French[11, 12] and German[13] groups have found that a normal or decreased dye arrival time rules out extrahepatic obstruction but that there are a few cases of delayed arrival time where no extrahepatic obstruction could be found. It is possible that spasm of the sphincter of Oddi may cause increased pressure in the biliary

system and simulate moderate extrahepatic obstruction, and that local irritation of the sphincter by a duodenal tube may produce spasm.[18] We have thus far found no such cases; whether this is due to improved technique or to our small series of patients is yet to be determined.

The data presented would seem to justify the following conclusions:

1. A semilog plot of data obtained from external scintillation counting rates at the head after the intravenous injection of radioactive rose bengal corresponds to rates of change in the blood concentration of dye.

2. Data obtained by counting over the liver in the first 2 to 10 minutes corresponds to the rate of uptake of dye by the polygonal cells (as does the mirror image of the head curve during the same time period). The head curve would seem to be a more accurate reflector of the liver uptake since its rate of change is not reduced by early excretion of the dye into the common duct and gall bladder.

3. Data obtained by counting over the lower abdomen corresponds to rates of change of dye concentrations in the abdominal blood and in the intestinal lumen.

4. The difference between the head and LLQ curves starting at the same point will represent the net rate of intestinal dye accumulation, and the point of divergence of these two curves from an essentially parallel course represents the arrival time of dye in the intestine.

5. When incomplete obstruction is present (sufficient dye reaching the intestine to be easily observable as a divergence of the head and LLQ curves), a delay in intestinal arrival time beyond 30 minutes suggests extrahepatic obstruction, while an arrival time of less than 20 minutes suggests intrahepatic obstruction.

6. When essentially no dye arrives in the intestine (complete obstruction), as demonstrated by nearly parallel head and LLQ curves, a 10- to 2-minute liver counting ratio (or the reciprocal of the head count) of 1.13 or less suggests severe hepatic cell damage or malfunction; if this ratio is 1.20 or more, it suggests extrahepatic obstruction of less than about 7 weeks' duration.

Further patient as well as animal studies are being undertaken and simplifications in technique are being developed. At the present time it is felt that by the technique described the state of the liver cells and the degree of biliary tract obstruction can be approximated, and the obstructing lesion can be placed within or beyond the liver with considerable accuracy.

SUMMARY

After the intravenous injection of I^{131}-labeled rose bengal, serial external scintillation counting at the head, liver, and lower abdomen describe curves from which the following data may be obtained: (1) the rate of dye disappearance from the blood, (2) its rate of uptake by the liver, (3) its time of arrival in the duodenum, and (4) its accumulation in the intestine. Decreased rose bengal accumulation in the intestine occurs in biliary tract obstruction of any

etiology. In addition, extrahepatic obstruction is characterized by a delayed arrival time of dye in the intestine, while the arrival time in intrahepatic obstruction is normal or early. When obstruction is so severe that little dye appears in the intestine, a markedly depressed liver curve suggests primary injury or malfunction of the polygonal cells. In our series, this curve was not similarly depressed in extrahepatic obstruction up to 7 weeks' duration. Possible exceptions and directions for further study are discussed.

We are indebted to Dr. Benedict Cassen, Clinical Professor of Biophysics, University of California at Los Angeles, for his advice and helpful criticism in the graphic resolution of the data. We also wish to acknowledge the secretarial assistance of Esther Flaskerud.

REFERENCES

1. Mendeloff, A. I.: Fluorescence of Intravenously Administered Rose Bengal Appears Only in Hepatic Polygonal Cells, Proc. Soc. Exper. Biol. & Med. **70**: 556, 1949.
2. Oak Ridge Institute of Nuclear Studies, Medical Division, Midyear Report, Dec. 31, 1956, p. 4.
3. Blahd, W. H., and Nordyke, R. A.: The Blood Disappearance of Radioactive Rose Bengal. A Rapid Simple Test of Liver Function, Clin. Res. Proc. **5**: 40, 1957.
4. Taplin, G. V., Meredith, O. M., Jr., and Kade, H.: The Radioactive (I^{131}-Tagged) Rose Bengal Uptake-Excretion Test for Liver Function Using External Gamma Ray Scintillation Counting Techniques, J. LAB. & CLIN. MED. **45**: 665, 1955.
5. Rosenberg, C. A., Lee, N. D., and Martignoni, P.: The Use of Radioactive Rose Bengal as a Liver Function Test, Clin. Res. Proc. **4**: 39, 1956.
6. Lowenstein, J. M.: Radioactive Rose Bengal Test as a Quantitative Measure of Liver Function, Proc. Soc. Exper. Biol. & Med. **93**: 377, 1956.
7. Marshall, W., and Kozoll, D. D.: Liver Uptake and Blood Clearance Studies of Radioactive (I^{131}) Rose-Bengal, J. LAB. & CLIN. MED. **48**: 924, 1956.
8. Brown, C. H., and Glasser, O.: Radioactive (I^{131}-Tagged) Rose-Bengal Liver Function Test, J. LAB. & CLIN. MED. **48**: 454, 1956.
9. Englert, E., Jr., Burrows, B. A., and Inglefinger, Franz, J.: Quantitative Evaluation of Hepatic Uptake and Release of Radioactive Rose Bengal, Clin. Res. Proc. **5**: 209, 1957.
10. Wood, J. A., and Korst, D. R.: Clinical Studies With Radioiodine Rose Bengal Dye, Clin. Res. Proc. **5**: 210, 1957.
11. Caroli, J., Tanasoglu, Y., and Cohen, M.: Le Temps d'Apparition de la Bromesulfonephtaléine dans la Bile Recueillie per Tubage, Arch. Mal. App. Digestif. **41**: 623, 1952.
12. Caroli, J., and Tanasoglu, Y.: Le Temps d'Apparition de la Bromesulfonephtaléine dans la Bile; Nouveau Test pour le Diagnostic des Ictères Incomplets par Rétention et des Blocages Anicteriques de la Voie Principale, Semaine Hôp. Paris **29**: 591, 1953.
13. Volkheimer, G., and Brüshke, G.: Über Eine Einfache Methode zur Prüfung der Durchgangigkeit des Ductus Choledochus, Ztschr. ges. inn. Med. **11**: 811, 1956.
14. Roholm, K., and Krarup, N. B.: Histopathology of the Liver in Obstructive Jaundice Examined by Aspiration Biopsy, Acta med. scandinav. **108**: 48, 1941.
15. Lucké, B.: The Pathology of Fatal Epidemic Hepatitis, Am. J. Path. **20**: 471, 1940.
16. Williams, W., and Greenberg, A.: Cytoplasmic Reactions of Hepatic Parenchymal Cells to Experimental Injury in Rodents and Dogs, Fed. Proc. **9**: 348, 1950.
17. Movitt, E. R., Meyer, M. A., Snell, A. M., Goldman, M. J., Gibson, J. R., Sullivan, B. H., Jr., Webster, J. G., and Stone, R. B.: Jaundice Associated With the Administration of Chlorpromazine, SKF-2601-A (Thorazine), Gastroenterology **28**: 901, 1955.
18. Newman, H. F., and Northup, J. D.: Hydrodynamics of the Human Common Duct, Surg., Gynec. & Obst. **105**: 355, 1957.

THE EFFECT OF HYDROCORTISONE AND DESOXYCORTICOSTERONE ON EXPERIMENTAL INFECTIONS WITH M. TUBERCULOSIS IN RABBITS

RICHARD L. SCHULTZ, PH.D.,* AND MABEL S. OKAWAKI, M.S.**
DENVER, COLO.

WITH THE TECHNICAL ASSISTANCE OF JACK A. MOORMAN, HAROLD L. SEERVELD, JEANNE LACERTE, AND JAMES S. MULLINS

THE over-all effects of steroid therapy on tuberculosis infections are not clear. There is considerable evidence that cortisone alone will adversely affect tuberculosis in experimental animals and man. The results are somewhat in question, possibly due in part to a variation in response to the hormone in different species.[1]

Most of the reported studies have involved the use of cortisone in tuberculosis, while the other adrenocortical hormones have not been investigated. This paper presents the results from an investigation into the effects of hydrocortisone and desoxycorticosterone on experimental infections with *M. tuberculosis* in rabbits. It also includes data concerning the effects of the infection and hormone treatment on the blood.

MATERIALS AND METHODS

Thirty female New Zealand white rabbits (2 to 4 kilograms) were injected intravenously with *M. tuberculosis* var. *Bovine vallee* at a concentration of approximately 1.4 × 10^6 cells per animal. The infected animals were divided into 3 experimental groups of 10. One group was given 0.1 ml. corn oil daily intraperitoneally; a second group was injected daily with 2.5 mg./Kg. hydrocortisone† in corn oil; and the last group received 2.5 mg./Kg. desoxycorticosterone† in corn oil daily.

Blood samples were obtained by cardiac puncture 2½ and 5 weeks after the animals were infected. Aliquots of the whole blood were analyzed for hemoglobin with the acid hematin method,[2] for hematocrit and sedimentation rate according to the method given by Wintrobe and Landsberg,[3] and a complete blood count was made using standard laboratory methods. Serum was obtained from aliquots of the whole blood by centrifugation at 2,500 r.p.m. for 10 minutes. The serum was analyzed for sodium and potassium with the flame photometer,[4] for chloride with the method outlined by Schales and Schales,[5] for blood urea nitrogen (BUN),[6] for total protein with a modification of the biuret method,[7] and for the relative concentration of the serum proteins using the Spinco Model R electrophoresis equipment consisting of Durrum "hanging strip" type cell and a recording and automatically integrating densitometer.[8] The "t" test was used for significance to compare the hormone treated animals with their oil controls.

From the U. S. Army Research and Development Unit, Fitzsimons Army Hospital, Denver, Colo.

Received for publication Sept. 30, 1957.

*Present Address: Department of Biology, University of Oregon, Eugene, Ore.

**Present Address: University of Nebraska School of Medicine, Omaha, Neb.

†The hormones were generously donated by Dr. Robert O. Stafford, Department of Endocrinology, The Upjohn Company, Kalamazoo, Mich.

The degree of infection was determined by autopsy at 5 weeks following the infection. One part of each lung was placed in 12.5 per cent trisodium phosphate and homogenized with a Virtis homogenizer for 10 minutes. The suspension was placed on a mechanical shaker for 7 minutes and incubated overnight at 37° C. The suspension was then centrifuged at 3,500 r.p.m. for 15 minutes and smears were made of the concentrate. The sediment was resuspended in Sorenson's buffer, pH 6.6,[9] and recentrifuged at 3,500 r.p.m. for 15 minutes. The supernatant was poured off and 10 c.c. of the buffer was added to the concentrate. Dilutions were made of aliquots of the suspension with distilled water. One milliliter of each dilution was added in duplicate to plates containing RDM-1 medium.[9] The plates were sealed in plastic bags, incubated in an upright position at 37° C., and the number of colonies of bacilli were counted after incubation periods of 3 and 4 weeks.

Pieces of the lung, spleen, and liver were fixed in 10 per cent formalin. For histopathologic examination, sections of these tissues were stained with Harris' hematoxylin and eosin stain and with Ziehl-Nielson acid alcohol stain.

The normal blood picture was obtained by drawing blood from 98 noninfected female rabbits, ranging in weight from 2 to 4 kilograms. The same analyses were done on these bloods as on the experimental groups. The whole blood determinations were done on bloods from 69 of these animals and the serum determinations were done on all 98. The standard deviation (σ) was determined for each analysis and a range established on the basis of a 2 σ spread.

An attempt was made to adrenalectomize rabbits in order to determine the effect of adrenalectomy on *M. tuberculosis* infections in this animal. A one-stage bilateral adrenalectomy was performed on 35 rabbits under sterile conditions. The approach was ventral and the adrenals were tied off from their blood supply with surgical thread before removal. Despite pre- and postoperative administration of varying concentrations of adrenal cortical extract * hydrocortisone acetate,† and/or desoxycorticosterone acetate,‡ only 3 rabbits lived more than a week following surgery. Therefore, the data collected on the effect of adrenalectomy on *M. tuberculosis* infections are insufficient.

TABLE I. A SUMMARY OF THE CONCENTRATION OF TUBERCLE BACILLI IN SMEARS AND CULTURES OF THE CONCENTRATE OBTAINED FROM LUNG HOMOGENATES

	SMEARS (APPROX. CONCENTRATION OF BACILLI OBSERVED)			CULTURES (NO. COLONIES/GM. TISSUE)			
	NONE	FEW	MANY	<10	10-100	100-1,000	>1,000
Oil controls	3	3	4	2	7	0	1
Hydrocortisone	0	1	7	1	1	3	3
Desoxycorticosterone	4	3	3	5	3	2	0

RESULTS

The Effect of the Hormone Treatment on the Severity of Infection.—A summary of the results from the smears and cultures of the concentrates prepared from the homogenates of the lungs is found in Table I. The observations on the smears were arbitrarily divided into groups on the basis of many, a few, and no bacilli observed. The results from the cultures were arbitrarily divided according to the number of colonies per gram wet weight of the original tissue. The results varied considerably, but the general trend showed that more bacilli were demonstrated in the lungs of the animals treated with hydrocortisone than in those of the desoxycorticosterone-treated or oil-treated animals.

*Parke, Davis & Company, Detroit, Mich.
†Sharp & Dohme, Inc. (Merck Sharp & Dohme), Philadelphia, Pa.
‡Organon, Inc., Orange, N. J.

TABLE II. THE EFFECT OF INFECTIONS WITH M. TUBERCULOSIS AND SUBSEQUENT TREATMENT WITH HYDROCORTISONE (F) AND DESOXYCORTICOSTERONE (DC) ON THE HEMOGLOBIN CONCENTRATION, HEMATOCRIT, AND COMPLETE BLOOD COUNT IN RABBITS

	NORMAL (69)*		2½ WEEKS POSTINFECTION			5 WEEKS POSTINFECTION		
	RANGE (2σ)	MEAN	CONTROLS (10)	F (10)	DC (10)	CONTROLS (10)	F (8)	DC (10)
Hemoglobin (Gm. %) mean	10.2-15.0	12.6	12.6	11.6	12.4	13.2	13.1	12.9
per cent in normal range		95.7	100	100	87.5	100	75	100
Hematocrit (% R.B.C.) mean	36.2-48.8	42.5	42.0	40.1	42.0	45.0	45.0	45.0
per cent in normal range		92.8	100	100	100	80	50	90
R.B.C. (millions) mean	3.06-6.68	4.87	5.00	4.77	4.91	6.04	5.61	5.43
per cent in normal range		92.8	100	100	100	90	100	100
W.B.C. (total) mean	3,523-12,619	8,071	7,450	4,550†	6,312	9,065	4,168†	8,985
per cent in normal range		95.7	100	55.5	100	90	87.5	90
Neutrophils (%) mean	0-28.3	10.7	7.6	17.7	12.3	22.2	17.1	10.3
per cent in normal range		92.8	100	77.8	87.5	70	75	100
Lymphocytes (%) mean	34.1-100	68.0	62.0	45.9	62.0	55.3	36.9	60.4
per cent in normal range		97.1	100	77.8	100	100.0	50.0	80
Monocytes (%) mean	0-4.9	2.2	4.7	4.9	5.5	4.9	2.9	4.9
per cent in normal range		92.8	55.6	55.5	37.5	60	75	40
Eosinophils (%)‡ mean	0-37.8	18.0	19.5	26.9	19.3	16.9	41.3	23.0
per cent in normal range		97.1	100	66.7	87.5	90	50	80
Basophils (%) mean	0-3.2	1.1	2.0	1.8	2.3	1.3	3.8	2.2
per cent in normal range		97.1	100	100	87.5	100	87.5	90

*Numbers in parentheses indicate number of animals.
†A significant difference when compared with oil-treated control.
‡This includes the pseudoeosinophils.

The 10 oil-treated animals all showed some degree of infection. Histologically, all of the lung sections showed extreme congestion and infiltration with a number of lesions, both hard and soft, with varying degrees of hyalinization of the large lesions. No acid-fast bacilli were observed in any of the tissues. All but one of the liver sections appeared normal; the one exception had numerous small areas of hyalinization and infiltration of giant cells and epithelioid cells. All of the spleens showed some degree of infection: 7 had a few small lesions and 3 had extensive large lesions.

The animals treated with hydrocortisone showed signs of the disease before autopsy. Two of the animals died before the sacrifice 5 weeks after the infection, and the remainder showed a loss in weight and a distinct change in physical appearance and activity. The livers of all of these animals consisted of vacuolated cells. The spleens generally showed extensive lesions in the white pulp, with some degree of hyalinization, and smaller lesions in the red pulp. The lungs were filled with lesions. Comparing the lungs with those of the oil controls, there was more caseous necrosis and the infection was generally more severe, both in dissemination and necrosis, with hydrocortisone treatment. There were many tubercle bacilli in all of the lung sections, many in one of the spleens, and a few in 5 of the other spleens, and one of the liver sections contained a few bacilli.

The severity of infection with the desoxycorticosterone was quite variable. It was more severe than the controls considering the liver and the spleen, but far less disseminated than with hydrocortisone. There was not the necrosis observed with hydrocortisone, but epithelioid cells were observed. Three of the livers showed lesion formation and 7 of the spleens showed extensive replacement of the white pulp with lesions. Acid-fast bacilli were observed in one of the lung sections but in none of the liver or spleen sections.

The Effects of Infection and Subsequent Hormone Treatment on the Blood.—The effect of the infection and subsequent treatment with the hormones on hemoglobin concentration, hematocrit, and complete blood count is shown in Table II. The hemoglobin concentration, hematocrit, and red blood count remain relatively stable under all experimental conditions. The total leukocyte count dropped significantly with hydrocortisone treatment but not in the oil- or desoxycorticosterone-treated animals.

There was little change in the differential count of the untreated infected animals except for a slight elevation in the per cent neutrophils and monocytes. With hydrocortisone administration for 2½ weeks, there was an increase in the per cent neutrophils, eosinophils, and monocytes, and a decrease in the per cent lymphocytes. After 5 weeks' treatment with hydrocortisone, the per cent lymphocytes decreased further, and the per cent eosinophils increased. The desoxycorticosterone treated animals showed a differential count similar to the untreated animals except there was no increase in the per cent neutrophils.

The results with the electrolytes and BUN are shown in Table III. All of these are relatively stable under the experimental conditions. The BUN is

TABLE III. EFFECTS OF INFECTIONS WITH M. TUBERCULOSIS AND SUBSEQUENT TREATMENT WITH HYDROCORTISONE (F) AND DESOXYCORTICOSTERONE (DC) ON THE BLOOD ELECTROLYTES AND BLOOD UREA NITROGEN (BUN) IN RABBITS

	NORMAL (98)*		2½ WEEKS POSTINFECTION			5 WEEKS POSTINFECTION		
	RANGE (2σ)	MEAN	CONTROLS (10)	F (10)	DC (10)	CONTROLS (10)	F (8)	DC (10)
BUN (mg. %) mean	9.0-27.8	18.4	17.0	17.8	11.6†	16.0	19.9	15.6
per cent in normal range		99	100	90	90	100	87.5	90
Cl (mEq./L.) mean	105.1-127.5	116.3	113.0	107.8	110.9	113.4	109.7	112.6
per cent in normal range		94.2	80	55.5	75	80	85.7	80
Na (mEq./L.) mean	133.0-158.4	145.7	148.0	146.2	150.1	151.1	153.5	153.1
per cent in normal range		94.9	100	80	90	90	87.5	90
K (mEq./L.) mean	3.3-5.8	4.6	4.5	4.8	3.9	4.3	5.0*	4.4
per cent in normal range		95.9	100	100	80	100	87.5	90

*Numbers in parentheses indicate number of animals.
†A significant difference when compared with oil-treated controls.

TABLE IV. EFFECTS OF INFECTIONS WITH M. TUBERCULOSIS AND SUBSEQUENT TREATMENT WITH HYDROCORTISONE (F) AND DESOXYCORTICOSTERONE (DC) ON THE TOTAL PROTEIN AND SERUM PROTEIN PATTERNS IN RABBITS

	NORMAL (98)*		2½ WEEKS POSTINFECTION			5 WEEKS POSTINFECTION		
	RANGE (2σ)	MEAN	CONTROLS (10)	F (10)	DC (10)	CONTROLS (10)	F (8)	DC (10)
Total protein (Gm. %) mean	5.14-7.26	6.20	6.08	6.81*	6.21	6.40	6.94	6.80
per cent in normal range		95.9	90	80	80	100	50	80
Albumin (% area) mean	52.30-83.10	67.70	57.38	64.14	58.74	56.25	53.92	51.88
per cent in normal range		94.8	80	90	100	60	50	50
Alpha-1 globulin (% area) mean	2.71-9.35	6.03	5.24	4.47	6.18	6.35	5.20	6.09
per cent in normal range		94.8	100	80	80	70	100	70
Alpha-2 globulin (% area) mean	1.74-8.90	5.32	7.18	6.25	6.05	6.39	6.04	6.04
per cent in normal range		96.9	80	80	90	90	87.5	90
Beta globulin (% area) mean	4.46-15.34	9.90	16.09	15.86	15.64	14.47	19.75†	17.55
per cent in normal range		96.9	50	60	60	60	12.5	30
Gamma globulin (% area) mean	9.60-26.60	18.10	14.10	9.28	13.38	16.55	15.08	18.44
per cent in normal range		94.8	70	30	70	90	75	90

*Numbers in parentheses indicate number of animals.
†A significant difference when compared with oil-treated controls.

significantly lower after 2½ weeks of treatment with desoxycorticosterone, but returns to normal after 5 weeks.

The results from the total protein and serum protein pattern determinations are presented in Table IV. The total protein shows a significant elevation with hydrocortisone treatment. The albumin showed a fairly consistent drop, especially after 5 weeks of infection, in all experimental groups. The alpha globulins showed little change. An elevation of the beta globulins was observed with all treatments, the most pronounced elevation shown after hydrocortisone. The hydrocortisone caused a drop in gamma globulin at 2½ weeks which returned toward normal at 5 weeks postinfection.

The sedimentation rate was consistently 1 mm./hr. except for an increase to 3, 5, and 12 mm./hr. in 3 animals treated with hydrocortisone.

DISCUSSION

The observed increase in the severity of the infection with hydrocortisone is similar to that reported with cortisone in rabbits.[10] This increase with hydrocortisone, in contrast to the relatively slight effect with desoxycorticosterone, would seem to agree with Selye's observations[11] that a high dose of glucocorticoids stimulate the inflammatory reactions, overcoming the buffering effect of an intact adrenal, while this stimulation above normal has proved to be extremely difficult with mineralocorticoids.

The increase in the beta globulin and decrease in albumin is similar to that reported by Seibert and Nelson[12] in experimentally infected tuberculous rabbits. Hudgins, Cummings, and Patnode[13] found an increase in gamma globulin and a decrease in albumin in rabbits following inoculation with the BCG strain of tubercle bacilli. Since the BCG inoculation has an immunizing effect on the animals, it appears that the gamma globulin is elevated in immunized rabbits while the beta globulin is elevated in the diseased animals.

The hormone treatments appear to have little effect on the serum protein patterns. Other workers have reported a lack of change in rabbit serum protein patterns following hydrocortisone and cortisone administration.[14, 15] The significant increase in the beta globulin after 5 weeks of hydrocortisone injections can be correlated with the increased severity of the disease. A decrease in gamma globulin following hydrocortisone administration in rats has been reported by Hoch-Ligeti and Irvine,[16] who also noted an increase in total protein in rats following a high dose of cortisone. A decrease in gamma globulin following cortisone treatment has also been observed in guinea pigs.[17]

A similar decrease in total leukocyte count was seen following cortisone injections in guinea pigs by Nicol and Bilbey.[18] These workers also noted a drop in the number of lymphocytes and a rise in pseudoeosinophils. The decrease in lymphocytes and total leukocytes, along with the initial drop in gamma globulin, is a reflection of the animals' decreased resistance to disease following hydrocortisone administration.

There were no apparent changes in the electrolyte concentration due to the hormone treatments. Generally, it has been demonstrated that effects of the

adrenal hormones are somewhat transitory and the concentrations will return to normal with continued administration of the hormone.[19]

SUMMARY

1. Hydrocortisone administration, following the infection of intact rabbits with *M. tuberculosis*, caused a marked increase in the severity of the disease, while desoxycortisone treatment caused little change in the infection when compared with the controls.

2. The infection effected an increase in the beta globulin and a decrease in the albumin. The hormone treatments induced little change in serum proteins except for an increase in the total protein and a transitory decrease in gamma globulin with hydrocortisone.

3. Little change was noted in the blood electrolyte content following the infection and subsequent treatment with hydrocortisone and desoxycorticosterone.

4. No changes were observed in the hemoglobin, hematocrit, erythrocyte count, and leukocyte count after the infection with the controls or the desoxycorticosterone treatment. Hydrocortisone caused a significant decrease in the leukocyte count.

We wish to express our thanks to Drs. Arthur F. Lincoln and William G. Cooper for their examination of the slides; to Martha Harrison for the preparation of the slides; to Dr. J. Harold Harrison for the many hours spent performing the adrenalectomies; and to Edward D. Jenkins for aid in the statistical analysis of the data.

REFERENCES

1. Johnson, J. R., and Davey, W. N.: Cortisone, Corticotropin, and Antimicrobial Therapy in Tuberculosis in Animals and Man, Am. Rev. Tuberc. **70**: 623, 1954.
2. Cohen, B., and Smith, A. H.: The Colorimetric Determination of Hemoglobin, J. Biol. Chem. **39**: 489, 1919.
3. Wintrobe, M. M., and Landsberg, J. W.: Standardized Technique for the Blood Sedimentation Test, Am. J. Med. Sc. **189**: 102, 1935.
4. Flame Photometer Manual, Baird Associates, Inc., Cambridge, Mass.
5. Schales, O., and Schales, S. S.: A Simple and Accurate Method for the Determination of Chloride in Biological Fluids, J. Biol. Chem. **140**: 879, 1941.
6. Gentzkow, C. J.: An Accurate Method for the Determination of Blood Urea Nitrogen by Direct Nesslerization, J. Biol. Chem. **143**: 531, 1942.
7. Cohn, C., and Wolfson, W. Q.: Studies in Serum Proteins. II. A Rapid Clinical Method for the Accurate Determination of Albumin and Globulin in Serum or Plasma, J. Lab. & Clin. Med. **33**: 367, 1948.
8. Spinco Model R Paper Electrophoresis System Operating Instructions, Spinco Division, Beckman Instruments, Inc., Belmont, Calif.
9. Taylor, R. L., Weiser, O. L., and Lincoln, A. F.: Laboratory Methods for Tuberculosis Bacteriology, Report No. 1 Revised, U. S. Army Research and Development Unit, 1957, Fitzsimons Army Hospital, Denver, Colo.
10. Lurie, M. B., Zappasodi, P., Dannenberg, A. M., and Swartz, I. B.: Constitutional Factors in Resistance to Infection: The Effect of Cortisone on the Pathogenesis of Tuberculosis, Science **113**: 234, 1951.
11. Selye, H.: Stress and Disease, Science **122**: 625, 1955.
12. Seibert, F. B., and Nelson, J. W.: Electrophoresis of Serum. Serum Proteins in Tuberculosis and Other Chronic Diseases, Am. Rev. Tuberc. **47**: 66, 1943.
13. Hudgins, P. C., Cummings, M. M., and Patnode, R. A.: Electrophoretic Distribution of Serum Proteins in Rabbit, Guinea Pig and Rat Following BCG Administration, Proc. Soc. Exper. Biol. & Med. **92**: 75, 1956.
14. Wang, C. I., Bossak, E. T., and Aldersberg, D.: Prednisone and Plasma Lipids, J. Clin. Endocrinol. & Metab. **15**: 1308, 1955.

15. Bossak, E. T., Wang, C. I., and Aldersberg, D.: Species Variations of Serum Proteins, Lipids and Lipoproteins to the Cortisones, J. Clin. Endocrinol. & Metab. **16**: 613, 1956.
16. Hoch-Ligeti, C., and Irvine, K.: Effects of Hormone Administration on Serum Protein Patterns, Proc. Soc. Exper. Biol. & Med. **87**: 324, 1954.
17. Snell, R. S., and Nicol, T.: Effect of Cortisone on the Serum Gamma-Globulin, Nature **177**: 578, 1956.
18. Nicol, T., and Bilbey, D. L. J.: Effect of Cortisone on Blood, Nature **177**: 524, 1956.
19. Hartman, F. A., and Brownell, K. A.: The Adrenal Gland. Philadelphia, 1949, Lea & Febiger.

OBJECTIVE EVALUATION OF PATIENTS WITH RHEUMATIC DISEASES

IV. Comparison of the Diphenylamine Reaction (DPA) With Serum Glycoprotein and Seromucoid Levels

M. R. Shetlar, Ph.D., and R. W. Payne, M.D.
Oklahoma City, Okla.

THE diphenylamine reaction, as originally described by Dische,[1] results in different colored reaction mixtures with different sugars, and thus has considerable value as a qualitative reagent in carbohydrate chemistry. When the reaction is carried out by heating the diphenylamine reagent with trichloroacetic acid filtrates of serum, a purple solution results. This reaction has recently been ascribed by Hess, Hahn, and Ayala[2] to the sialic acid found in the seromucoid fraction of serum. Niazi and State[3] noted that increased amounts of diphenylamine reactive material were present in the sera of patients with neoplasms, pulmonary tuberculosis, and rheumatic fever. This serum component has been further studied in patients with rheumatic fever by Ayala, Moore, and Hess,[4] and by Coburn, Moore, and Haninger.[5] Fearnley and co-workers[6] and Cecchi and Ferraris[7] compared the diphenylamine reaction (DPA) with the erythrocyte sedimentation rate as a means of measuring clinical activity in patients with rheumatoid arthritis. As the serum glycoprotein has been shown to serve as an index of inflammatory activity in the rheumatic diseases,[8] the following study was concerned with the comparison of the diphenylamine reaction with serum glycoprotein and seromucoid concentrations in normal individuals and in patients with rheumatoid arthritis. In addition, patients with other conditions known to influence serum glycoprotein and seromucoid were included in the study.

METHODS

Laboratory.—Total serum glycoprotein was estimated by the method of Shetlar, Foster, and Everett,[9] seromucoid by the method of Weimer and Moshin,[10] and serum protein by the biuret method of Weischelbaum.[11] The diphenylamine reaction was modified slightly from that described by Ayala, Moore, and Hess.[4] To 0.4 milliliter of serum in a 18 by 150 mm. test tube, 10 ml. of 5 per cent trichloroacetic acid was added slowly with shaking. After placing marbles over the mouths of the tubes, they were placed in a boiling water bath for 20 minutes. Tubes were then cooled in an ice bath after which the contents were filtered through Whatman No. 1 filter paper. Three milliliters of the filtrate was transferred to each of 2 glass-stoppered test tubes (18 by 150 mm.) and 3 ml. of 5 per cent trichloroacetic

From the Departments of Biochemistry and Pharmacology, University of Oklahoma School of Medicine, Oklahoma City, Okla.

This investigation was supported in part by Research Grant A 818 from the National Institute of Arthritis and Metabolic Disease of the National Institutes of Health.

Received for publication Oct. 4, 1957.

acid was pipetted into a third glass-stoppered tube for use as a reagent blank. To one sample and to the reagent blank, 6 ml. of color reagent (1 Gm. of diphenylamine in 90 ml. glacial acetic acid and 10 ml. of concentrated sulfuric acid) was added. To the second sample tube, 6 ml. of acid solution (90 ml. glacial acetic and 10 ml. of concentrated sulfuric acids) was added; this tube served as the sample blank. Several glass helices were placed in each tube to prevent bumping, the tubes were then stoppered and the contents mixed by inversion. All tubes were placed in a boiling water bath for 30 minutes and then cooled in an ice bath for 10 minutes. Readings of absorbance were made at 530 mm. in a Coleman 14 spectrophotometer using square 13 mm. cuvettes. Samples were read against the reagent blank, while the sample blanks were read against a mixture of 3 ml. of 5 per cent trichloroacetic acid and 6 ml. of acid solution. The latter readings were deducted from the former to obtain a net absorbance for each sample. Results are expressed as absorbance obtained under these conditions. As no suitable primary standard was available for this test, an aliquot of a large pooled sample of human serum was included in each run as a reference.

Clinical.—Patients were selected for study from the Arthritis Clinic of the University Hospitals. An estimate of clinical activity of the rheumatoid process for each patient was made at each clinic visit according to criteria previously described.[12]

TABLE I. SUMMARY OF DIPHENYLAMINE REACTION, SEROMUCOID, SERUM GLYCOPROTEINS, AND SERUM PROTEIN LEVELS

	NO. PATIENTS	DIPHENYLAMINE REACTION	SERO-MUCOID*	SERUM (MG. %)	GLYCOPROTEIN* (PR)	SERUM PROTEIN
Normal	9	0.208 ± 0.008	12 ± 1.1	120 ± 3.3	1.63 ± 0.04	7.37
		(0.170 – 0.240)	(9–17)	(102–133)	(1.43 – 1.79)	(6.80–7.69)
Rheumatoid arthritis						
Inactive (Grade I)	11	0.240 ± 0.009	15 ± 0.5	133 ± 3.3	1.83 ± 0.03	7.27
		(0.202 – 0.273)	(12–17)	(108–149)	(1.72 – 1.98)	(6.54–8.15)
Active (Grade II)	20	0.289 ± 0.010	24 ± 1.3	170 ± 1.3	2.28 ± 0.05	7.43
		(0.222 – 0.358)	(12–35)	(140–212)	(1.95 – 2.39)	(6.54–8.04)
Active (Grade III)	17	0.307 ± 0.011	29 ± 1.3	196 ± 4.7	2.54 ± 0.03	7.72
		(0.248 – 0.389)	(19–39)	(173–242)	(2.39 – 2.65)	(6.80–9.20)
Active (Grade IV)	4	0.360	34	208	3.20	6.58
		(0.293 – 0.456)	(15–49)	(180–222)	(2.88 – 3.63)	(4.96–7.30)
Carcinoma	8	0.298 ± 0.018	33 ± 3.6	189 ± 7.5	2.68 ± 0.15	6.86
		(0.229 – 0.381)	(16–44)	(154–212)	(2.15 – 3.17)	(5.86–7.85)
Multiple myeloma	1	0.472	17	354	4.08	8.69
Pregnant	2	0.254	13	160	2.40	6.70
Fetal	1	0.141	7	85	1.38	6.16

*Bound hexose as determined by the tryptophane method.[9] Hexosamine component of polysaccharide complex is not included.

Figures following ± are standard errors of the mean of each group. Figures in parentheses are the range for each group.

RESULTS

The concentrations of various serum components present in patients with rheumatoid arthritis or with various neoplasms, as well, as in pregnant and normal individuals are summarized in Table I. In confirmation of previous work[6, 7] sera from patients with rheumatoid arthritis were found to contain elevated diphenylamine reactive material; the concentration of this material generally paralleled clinical activity of the disease. Elevated diphenylamine

reactive material was also noted in patients with carcinoma and in one case of multiple myeloma. Two samples from pregnant subjects exhibited only slight elevations of this material, while one sample of fetal serum was decidedly lower than adult samples.

Correlation coefficients between diphenylamine, seromucoid, and glycoprotein levels and clinical activity of the patients with rheumatoid arthritis are summarized in Table II. The diphenylamine reaction exhibited a significant correlation with clinical activity. However, the correlation coefficients between clinical activity and either seromucoid or glycoprotein levels were significantly higher (at 4 per cent level for seromucoid, at the 0.06 per cent level for glycoprotein) than that between clinical activity and DPA. Expressing the results as a ratio to serum protein did not result in higher correlation coefficients with clinical activity for DPA and seromucoid, but did significantly improve the glycoprotein correlation (at the 5 per cent level of probability). The DPA results were found to be significantly correlated with both seromucoid and glycoprotein-bound hexose (Table II).

TABLE II. SUMMARY OF CORRELATION COEFFICIENT DERIVED FROM STUDIES OF PATIENTS WITH RHEUMATOID ARTHRITIS

	r*	"t" VALUE†	P
Correlation With Clinical Activity			
Diphenylamine reaction (DPA)	0.588	5.14	0.01
DPA ÷ serum protein	0.417	3.24	0.01
Seromucoid‡ (mg. %)	0.740	7.78	0.01
Seromucoid ÷ serum protein	0.673	6.43	0.01
Glycoprotein‡ (mg. %)	0.818	9.97	0.01
Glycoprotein ÷ serum protein (PR)	0.891	13.88	0.01
Correlation With DPA			
Seromucoid (mg. %)	0.679	6.54	0.01
Glycoprotein (mg. %)	0.720	7.28	0.01
Glycoprotein ÷ serum protein (PR)	0.499	4.04	0.01

*r = correlation coefficient.

†$t = \frac{n^{1/2}r}{(1-r^2)^{1/2}}$; n = N − 2.

‡Expressed as bound hexose.

DISCUSSION

All of the laboratory tests included in this study are of value as objective means of following the inflammatory activity of rheumatoid arthritis. The determination of glycoprotein and the diphenylamine reaction present approximately equal technical difficulty while seromucoid determinations are more difficult. The diphenylamine reaction currently has the disadvantage in that it has no readily available standard. Use of a suitable standard would increase the accuracy of the test and might result in a better correlation to clinical activity.

The relationships of the various tests to each other are of considerable interest. If the diphenylamine reaction in serum measures largely sialic acid and if this constituent is found only or mainly in the seromucoid fraction a high correlation coefficient should be found between DPA and seromucoid. This may indicate that sialic acid occurs in serum protein-polysaccharide complexes

other than seromucoid. The possibility also exists that the sialic acid content of the seromucoid subfractions varies and that one subfraction is more affected by the rheumatic process than are others.

SUMMARY

The diphenylamine reaction (DPA) in serum has been compared with serum glycoprotein and seromucoid determinations as a means of estimating the inflammatory activity in patients with rheumatoid arthritis. Although a significant correlation was found between DPA and clinical activity of such patients, the correlation was not as good as that found between either serum glycoprotein or seromucoid levels and clinical activity.

REFERENCES

1. Dische, S.: Über Microbestimmung der Kohlehydrate in tierischen Organen und in Blute mit Hilfe Charakterstische Farbreaktionen Microchemie **7**: 33, 1929.
2. Hess, E. L., Hahn, J. W., and Ayala, W.: Structures Responsible for the Characteristic Purple Color Development in Diphenylamine Reaction With Serum, Proc. Soc. Exper. Biol. & Med. **91**: 528, 1956.
3. Niazi, S., and State, D.: The Diphenylamine Reaction of Human Serum, Cancer Res. **8**: 653, 1948.
4. Ayala, W., Moore, L. V., and Hess, E. L.: The Purple Color Reaction Given by Diphenylamine Reagent. I. With Normal and Rheumatic Fever Sera, J. Clin. Invest. **30**: 781, 1951.
5. Coburn, A. F., Moore, L. V., and Haninger, J.: Serum Diphenylamine Reaction in Rheumatic Fever, A. M. A. Arch. Int. Med. **92**: 185, 1953.
6. Fearnley, G. R., Pirkis, J., De Coek, N., Lackney, R., and Meanock, R. I.: Diphenylamine Reaction in Rheumatoid Arthritis, Ann. Rheumat. Dis. **14**: 226, 1955.
7. Cecchi, E., and Ferraris, F.: Serum Diphenylamine Reaction in Rheumatoid Arthritis, Ann. Rheumat. Dis. **14**: 267, 1955.
8. Payne, R. W., Shetlar, M. R., Bullock, J. A., Patrick, D. R., Hellbaum, A. A., and Ishmael, W. K.: The Serum Polysaccharide Protein Ratio (PR) as a Measure of Rheumatoid Activity, Ann. Int. Med. **41**: 775, 1954.
9. Shetlar, M. R., Foster, J. V., and Everett, M. R.: Determination of Serum Polysaccharide by the Tryptophan Reaction, Proc. Soc. Exper. Biol. & Med. **67**: 125, 1948.
10. Weimer, H. E., and Moshin, J. R.: Serum Glycoprotein Concentrations in Experimental Tuberculosis of Guinea Pigs, Ann. Rev. Tuberc. **68**: 594, 1953.
11. Weichselbaum, T. E., and Shapiro, I.: An Accurate and Simple Method for the Determination of Proteins in Small Amounts of Blood Serum and Plasma, Am. J. Clin. Path. **10**: 40, 1946.
12. Stidworthy, G., Payne, R. W., Shetlar, C. L., and Shetlar, M. R.: Objective Evaluation of Patients With Rheumatic Diseases. II. Paper Electrophoretic Studies of Serum Glycoprotein and Protein From Patients With Rheumatoid Arthritis, J. Clin. Invest. **36**: 309, 1957.

PERIPHERAL BIOCHEMICAL CHANGES ASSOCIATED WITH THE INTRAVENOUS ADMINISTRATION OF AMMONIUM SALTS IN NORMAL SUBJECTS

MALCOLM P. TYOR, M.D., AND WILLIAM P. WILSON, M.D.
DURHAM, N. C.

THE role of ammonia in the production of certain clinical syndromes, particularly hepatic coma, has been a subject of recent investigation by several groups.[1-4] It is apparent that peripheral and cerebral uptake of ammonia may occur in patients with hepatic failure in the presence of an elevated arterial ammonia concentration and that the blood ammonia concentration is modified by the degree of this uptake.[5, 6] The peripheral uptake of ammonia in such patients may be quite variable.[6, 7]

It is the purpose of the present study to describe observations made on the peripheral uptake of ammonia in 9 normal subjects, following the intravenous administration of ammonium salts, with particular reference to the variability of this uptake and its relationship to continued intravenous loading.

METHOD

The 9 subjects were hospitalized men without clinical or laboratory evidence of hepatic or cardiovascular renal disease. The age range was from 25 to 46 years. All observations were made with the subjects recumbent after a rest period of 10 to 15 minutes. Thirty-seven to 94 mEq. of NH_3 as 0.155 M ammonium lactate (6 subjects) or 0.155 M ammonium chloride (3 subjects) was infused intravenously over a one-hour period, at rates varying between 0.007 to 0.018 mEq./min./Kg. for the first 30 minutes and 0.014 to 0.025 mEq./min./Kg. for the second 30 minutes. Arterial and venous blood (withdrawn without stasis) was obtained from a brachial artery and a medial subcutaneous antecubital vein of the same arm. Samples were taken before infusion, at 30 and 60 minutes after initiation of infusion, and at 15 minutes after discontinuation. Arterial and peripheral venous ammonia concentrations were determined in an additional 35 patients, who had a variety of disorders but no evidence of liver disease.

The blood ammonia (NH_3) was measured by a modification of the microdiffusion method of Brown and associates.[8] A 1 ml. aliquot of whole blood was introduced into the diffusion bottle from a calibrated heparinized tuberculin syringe 20 minutes after shedding and rotated at 78 r.p.m. for 30 minutes. The ammonia concentration was determined, using a Coleman senior spectrophotometer, with sodium hypochlorite as the indicator. Duplicate samples, blanks, and standards were run simultaneously and all values were calculated on the basis of these standards. The accuracy of this method from the standard deviation from mean recoveries was ± 3 per cent, using standards and blanks. The maximum difference of duplicate samples obtained from separate syringes was 10 per cent. Preliminary

From the Departments of Medicine and Psychiatry, Duke University School of Medicine, and the Veterans Administration Hospital, Durham, N. C.
Aided in part by Grant M 1203, National Institute of Mental Health.
Received for publication Oct. 4, 1957.

studies did not reveal significant differences of ammonia concentration of: (1) blood samples simultaneously withdrawn into oxalated, citrated, or heparinized syringes; (2) the same blood sample introduced into diffusion bottles from 40 seconds to 90 minutes after shedding; (3) the same blood sample when rotated for periods up to 60 minutes at 78 r.p.m. The pH of whole blood was measured with a Cambridge Model R pH meter equipped with an enclosed glass electrode. Measurements were made at room temperature, usually 25° to 26° C., and the results corrected to 37° C. by Rosenthal's factor.[9] The CO_2 content of whole blood was determined by the method of Van Slyke and Neill; the plasma CO_2 content was calculated from this value and the pH and the hemoglobin concentration by the line chart of Van Slyke and Sendroy.[10, 11] The plasma CO_2 tension was calculated by the Henderson-Hasselbalch equation.[12] Serum potassium and sodium was measured with an internal standard flame photometer.[13] Blood glucose was measured by the method of Nelson.[14]

In the analysis of these data all subjects were considered as a group, irrespective of the ammonium salt administered. The differences between the means of paired values were compared.

RESULTS

Resting arterial ammonia concentrations ($\bar{d}$ = 68 μg/100 ml.) and peripheral A-V differences ($\bar{d}$ = + 3 μg/100 ml.) in 9 normal subjects were similar to values obtained in 35 patients with a variety of disorders; $\bar{d}$ = 78 ± 18 and − 5 ± 22 μg/100 ml., respectively.

The intravenous infusion of ammonium salts was sufficient to produce a progressive increase in arterial ammonia concentration at 30 and 60 minutes in all subjects.

The peripheral arterial venous differences of ammonia increased at 30 minutes, when compared with preinfusion values ($\bar{d}$ = + 0.13 mEq./L., $p = < 0.01$). However, 60-minute A-V differences showed no further change ($p = > 0.05$); a subsequent decline toward control values was observed.

Arterial potassium concentration increased at 30 minutes, when compared with control levels ($\bar{d}$ = + 0.38 mEq./L.; $p = < 0.01$). No further change was observed at 60 minutes ($p = > 0.50$). Postinfusion levels fell below 60-minute values in most instances. Arterial sodium concentration was unchanged throughout the study.

Arterial glucose concentration increased significantly at 30 minutes ($\bar{d}$ = + 5.8 mg. per cent, $p = < 0.01$). Sixty-minute and postinfusion levels did not show a consistent pattern.

There was no consistent change in the arterial pH or CO_2 content of subjects who were given ammonium lactate, nor in the arterial pH of subjects given ammonium chloride. The CO_2 content of the 3 subjects given ammonium chloride decreased at 60 minutes.

COMMENT

The present data confirm previous observations that resting subjects with normal arterial ammonia concentrations do not show significant peripheral arterial venous ammonia differences.[5, 6]

We have previously reported upon the uptake of ammonia in the periphery of normal subjects who were receiving intravenous infusions of ammonium

TABLE I. BIOCHEMICAL CHANGES FOLLOWING INTRAVENOUS INFUSION OF AMMONIUM SALTS

SUBJECT	PERIOD AND TIME (MIN.)	NH_3 INFUSED: TOTAL mEq.	NH_3 INFUSED: mEq./MIN./KG.	NH_3 (mEq./L.): ARTERIAL	NH_3 (mEq./L.): A-V	POTASSIUM (mEq./L.*)	GLUCOSE (mg. %*)	pH*	CO_2 CONTENT (VOL. %*)	pCO_2 (mm. Hg*)
1. H. L.†	Preinfus. 5	55		0.04	0.0	4.2	83	7.36	46.0	43
	Infusion 30		0.013	0.34	+0.28	4.4	91	7.36	45.5	43
	Infusion 60		0.018	0.39	+0.27	4.4	90	7.44	41.5	33
	Postinfus. 15			0.08	+0.02	3.9	83	7.36	45.5	43
2. H. D.†	Preinfus. 5	60		0.04	−0.01	4.2	65	7.33	49.7	50
	Infusion 30		0.012	0.21	+0.09	4.3	70	7.33	50.5	51
	Infusion 60		0.019	0.26	+0.09	4.3	79	7.40	46.2	41
	Postinfus. 15			0.08	−0.04	4.0	79	7.35	49.6	48
3. J. D.†	Preinfus. 5	63		0.05	+0.01	4.0	77	7.29	45.0	49
	Infusion 30		0.013	0.25	+0.10	4.8	78	7.26	45.7	53
	Infusion 60		0.020	0.39	+0.18	4.7	80	7.26	44.8	52
	Postinfus. 15			0.10	−0.01	4.7	81	7.29	44.9	49
4. A. G.†	Preinfus. 5	94		0.02	+0.01	4.1	87	7.36	45.7	43
	Infusion 30		0.012	0.36	+0.25	4.6	92	7.36	43.8	42
	Infusion 60		0.024	0.48	+0.27	4.6	92	7.35	44.2	43
	Postinfus. 15			0.12	+0.02	4.5	94	7.36	45.5	43
5. F. H.†‡	Preinfus. 5	78		0.04	0.0	4.0	76	7.37	47.2	44
	Infusion 30		0.016	0.37	+0.04	4.3	88	7.37	47.3	44
	Infusion 60		0.021			4.2		7.41	44.0	38

6. J. C.†§	Preinfus.	5	37		0.04	-0.02	4.1	80	7.37	48.1	44
	Infusion	30		0.007	0.13	+0.03	4.3	83	7.37	50.2	46
	Infusion	60		0.014	0.35	+0.18	4.5	86	7.36	49.3	46
7. F. C.‖	Preinfus.	5	72		0.05	0.0	3.7	75	7.37	49.1	46
	Infusion	30		0.018	0.39	+0.03	4.7	85	7.37	47.5	44
	Infusion	60		0.022	0.51	+0.04	4.4	79	7.36	46.2	44
	Postinfus.	15			0.09	-0.04	4.3	80	7.39	45.0	40
8. R. M.‖	Preinfus.	5	76		0.02	+0.01	3.8	76	7.37	45.6	42
	Infusion	30		0.017	0.28	+0.19	4.0	83	7.34	45.2	47
	Infusion	60		0.025	0.38	+0.24	4.2	78	7.39	40.5	36
	Postinfus.	15			0.05	0.0	4.0	72	7.33	43.8	44
9. W. B.‖	Preinfus.	5	75		0.06	+0.02	4.5	75	7.36	45.0	40
	Infusion	30		0.016	0.34	+0.17	4.6	76	7.36	42.4	38
	Infusion	60		0.023	0.48	+0.17	4.6	90	7.30	39.4	40
	Postinfus.	15			0.07	-0.05	4.3	88	7.30	39.9	40

•Arterial samples.
†Ammonium lactate.
‡Incomplete sampling necessitated by development of nausea and vomiting.
§Postinfusion samples not obtained.
‖Ammonium chloride.

salts.[15] This observation has been confirmed by Summerskill and associates.[6] In addition, the present data confirm these authors' observations that the peripheral uptake of ammonia in this situation tended to increase in relation to an increase in arterial concentration.[6] It is apparent, however, that an additional increase in arterial ammonia concentration may not be accompanied by a significant increase in peripheral uptake. Considerable individual variation exists. Indeed, two subjects (F. H. and F. C.), whose arterial concentration rose abruptly during the first 30 minutes of infusion, exhibited minimal peripheral uptake. The data suggest that the peripheral uptake of ammonia in normal subjects was initially related to arterial concentration; however, this mechanism was apparently readily saturated with increased loading. These findings suggest similar mechanisms of peripheral A-V ammonia uptake in our normal subjects, whose arterial ammonia level was increased acutely, and patients with hepatic failure. In such patients the magnitude of the peripheral A-V ammonia difference may be correlated with the height of arterial concentration early in hepatic failure; however, this correlation becomes less evident with prolonged elevations of arterial ammonia or with further increases resulting from an oral nitrogenous load or gastrointestinal hemorrhage.[5-7] Indeed, a negative A-V ammonia difference has been reported in some patients with hepatic coma, suggesting peripheral release of ammonia.[6] In the present study, minimal negative A-V differences were seen in 4 of 7 subjects observed during the postinfusion period.

Elevations of peripheral venous potassium concentration in anesthetized dogs following the intravenous infusion of acidifying ammonium salts have been reported by Roberts and associates and Keating and co-workers.[16, 17] These changes were attributed to an associated decrease in hydrogen-ion concentration by the latter authors.[17] However, Roberts and associates produced a similar reduction in pH with the infusion of hydrochloric acid, without a concomitant alteration in potassium concentration.[16] Her data suggest a relationship between the infusion of ammonium ion and potassium concentration uninfluenced by hydrogen-ion concentration. The modest increases in potassium concentration noted at 30 minutes in our subjects occurred following the administration of either ammonium lactate or ammonium chloride and were not associated with a significant or consistent change in arterial pH. The magnitude of the increase in arterial potassium concentration observed in individual subjects at 30 minutes was variable and could not be correlated with arterial ammonia concentrations or A-V differences. When the data were treated as a group, however, the rise and fall of potassium concentrations paralleled that of the A-V ammonia differences. The reported exchange of intracellular potassium for a basic amino acid in certain circumstances may have some bearing on this parallelism.[18, 19]

Although the initial elevations of potassium coincided with an increase in arterial glucose concentration, the magnitude of the rise in potassium and glucose did not correlate in individual subjects. It is possible that these changes may

be mediated by epinephrine or norepinephrine.[20-21] Epinephrine and norepinephrine measurements* in one subject (W. B., at 30 minutes) were not significantly changed from normal base-line values. In addition, arterial sodium concentrations were not altered in any subject throughout the study. An alternate mechanism for the observed potassium release and hyperglycemia may reside in the inhibitory effect of ammonia upon the citric acid cycle. This type of mechanism has been proposed by Recknagel and Potter for the ketogenic effect of ammonia on liver slices and by Bessman as the cerebral biochemical defect in hepatic coma.[22, 23] Malonate, a citric acid cycle inhibitor, causes both ketosis and hyperglycemia in vivo.[24] The role of energy yielding reactions in maintaining potassium concentration has been amply documented, and a decrease in oxidations mediated by the citric acid cycle may lead to potassium release.[25, 26] Although no previous observations have been reported on glucose concentration following the infusion of ammonium salts, marked hyperglycemia has been observed in rats and dogs following the administration of large amounts of amino acids.[27, 28] No explanation has been offered for these observations.

The respiratory alkalosis produced by the infusion of nonacidifying ammonium salts in unanesthetized dogs has been commented upon by Vanamee and associates.[29] In the present study, alterations in pH and CO_2 content consistent with a respiratory alkalosis were observed at 60 minutes in 3 of 5 subjects given ammonium lactate at similar rates of infusion. Gross hyperpnea was not observed in any subject, except F. H. who was nauseated at the 60-minute period. There is no evidence for a direct effect of ammonia on the respiratory center. A progressive decrease in cerebral oxygen utilization, without an associated decline in cerebral blood flow, has been demonstrated by Fazekas and co-workers in patients with hepatic coma.[30] Such patients nearly uniformly demonstrate a respiratory alkalosis and ammonemia.[29] There are no data dealing with cerebral oxygen utilization in normal subjects infused with nonacidifying ammonium salts. A depression of cerebral metabolism might be inferred from the finding of a 30 per cent decrease in the cerebral (sagittal sinus) A-V oxygen difference and a concomitant decrease in A-V CO_2 difference of 2 anesthetized dogs 60 minutes after the infusion of 0.08 mEq./min./Kg. of ammonium lactate.[31]

None of our subjects, except F. H., experienced toxic symptoms. Continuous electroencephalographic recordings were obtained in all subjects; none showed gross alterations.

SUMMARY

1. Nine normal subjects were given intravenous infusions of 37 to 94 mEq. of NH_3 as ammonium lactate or chloride.

2. Peripheral A-V ammonia differences increased 30 minutes after infusion was initiated; however, 60-minute A-V differences remained unchanged, despite

*Determinations made by Dr. John Wallace, Department of Medicine, Duke University School of Medicine, Durham, N. C.

a progressive increase in arterial ammonia concentration. Arterial potassium concentration also increased at 30 minutes and remained unchanged at 60 minutes.

3. These findings were associated with significant increases in arterial glucose at 30 minutes. They could not be explained by changes in arterial pH and CO_2 content.

4. Possible mechanisms for these findings were discussed.

We gratefully acknowledge the helpful criticism of Dr. Henry Kamin.

REFERENCES

1. Bessman, S. P.: The Role of Ammonia in Clinical Syndromes, Ann. Int. Med. **44**: 1037, 1956.
2. Phillips, G. B., Schwartz, R., Gabuzda, G. J., Jr., and Davidson, C. S.: Syndrome of Impending Hepatic Coma in Patients With Cirrhosis of the Liver Given Certain Nitrogenous Substances, New England J. Med. **247**: 239, 1952.
3. McDermott, W. V., Jr., Adams, R. D., and Riddell, H. G.: Ammonia Metabolism in Man, Ann. Surg. **140**: 539, 1954.
4. Phear, E. A., Sherlock, S., and Summerskill, W. H. J.: Blood Ammonia Levels in Liver Disease and "Hepatic Coma," Lancet **1**: 836, 1955.
5. Bessman, S. P., and Bradley, J. E.: Uptake of Ammonia by Muscle, Its Implications in Ammoniagenic Coma, New England J. Med. **253**: 1143, 1955.
6. Summerskill, W. H. J., Wolfe, S. J., and Davidson, C. S.: The Metabolism of Ammonia and α-Keto-acids in Liver Disease and Hepatic Coma, J. Clin. Invest. **36**: 361, 1957.
7. Tyor, M. P., and Sieker, H. O.: The Ammonia Arterial-Venous Difference in Hepatic Failure, as Influenced by Alterations in Peripheral Circulation, Clin. Res. Proc. (Abst.) **5**: 214, 1957.
8. Brown, R. H., Duda, G. D., Korkes, S., and Handler, P.: A Colorimetric Micromethod for Determination of Ammonia; the Ammonia Content of Rat Tissue and Human Plasma, Arch. Biochem. **66**: 301, 1957.
9. Rosenthal, T. B.: The Effect of Temperature on the pH of Blood and Plasma in Vitro, J. Biol. Chem. **173**: 25, 1948.
10. Van Slyke, D. D., and Neill, J. M.: The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurements, J. Biol. Chem. **61**: 523, 1924.
11. Van Slyke, D. D., and Sendroy, J., Jr.: Studies of Gas and Electrolyte Equilibria in Blood. XV. Line Charts for Graphic Calculations by the Henderson-Hasselbalch Equation, and for Calculating Plasma Carbon Dioxide Content From Whole Blood Content, J. Biol. Chem. **79**: 781, 1928.
12. Dill, D. B., Daly, C., and Forbes, W. H.: The pK′ of Serum and Red Cells, J. Biol. Chem. **117**: 569, 1937.
13. White, J. U.: Precision of a Simple Flame Photometer, Anal. Chem. **24**: 394, 1952.
14. Nelson, N. A.: A Photometric Adaptation of the Somogyi Method for the Determination of Glucose, J. Biol. Chem. **153**: 375, 1944.
15. Tyor, M. P., and Wilson, W. P.: Biochemical Changes Associated With the Intravenous Administration of Ammonium Salts in Normal Subjects, Clin. Res. Proc. **5**: 107, 1957.
16. Roberts, K. E., and Magida, M. G.: Electrocardiographic Alterations Produced by Increase in Potassium, Circ. Res. **1**: 206, 1953.
17. Keating, R. E., Weichselbaum, T. E., Alanis, M., Margraf, H. W., and Elman, R.: The Movement of Potassium During Experimental Acidosis and Alkalosis in the Nephrectomized Dog, Surg., Gynec. & Obst. **96**: 323, 1953.
18. Christensen, H. N., Riggs, T. R., Fischer, H., and Palatine, I. M.: Intense Concentration of α, γ-diaminobutyric Acid by Cells, J. Biol. Chem. **198**: 17, 1952.
19. Iacobellis, M., Muntwyler, E., and Dodges, C. L.: Free Amino Acid Patterns of Certain Tissues From Potassium and/or Protein-Deficient Rats, Am. J. Physiol. **185**: 275, 1956.
20. Brewer, G., Larson, P. S., and Schroeder, A. R.: On the Effect of Epinephrine on Blood Potassium, Am. J. Physiol. **126**: 708, 1939.
21. Muirhead, E. E., Goth, A., and Jones, F.: Sodium and Potassium Exchanges Associated With Nor-epinephrine Infusions, Am. J. Physiol. **179**: 1, 1954.
22. Recknagel, R. D., and Potter, V. R.: Mechanisms of Ketogenic Effect of Ammonium Chloride, J. Biol. Chem. **191**: 263, 1951.

23. Bessman, S. P.: Ammonia Metabolism in Animals, A Symposium on Inorganic Nitrogen Metabolism by McElroy and Glass, Johns Hopkins Press, 1956, p. 408.
24. Handler, P.: Effect of Various Inhibitors of Carbohydrate Metabolism, in Vivo, J. Biol. Chem. **161**: 53, 1945.
25. Tenner, C., Eggleston, L. V., and Krebs, H. A.: The Role of Glutamic Acid in the Transport of Potassium in Brain and Retina, Biochem. J. **47**: 139, 1950.
26. Mudge, G. H.: Studies on Potassium Accumulation by Rabbit Kidney Slices: Effect of Metabolic Activity, Am. J. Physiol. **165**: 113, 1951.
27. Winetz, M., Gullino, P., Greenstein, J. P., and Birnbaum, S. M.: Studies in the Metabolism of Amino Acids and Related Compounds in Vivo. II. Effect of Toxic Doses of Essential Amino Acids on Blood Sugar, Liver Glycogen and Muscle Glycogen Levels, Arch. Biochem. **64**: 333, 1956.
28. Handler, P., Kamin, H., and Harris, J. S.: Metabolism of Parenterally Administered Amino Acids; Glycine, J. Biol. Chem. **179**: 283, 1949.
29. Vanamee, P., Poppell, J. W., Glicksman, A. S., Randall, H. T., and Roberts, K. E.: Respiratory Alkalosis in Hepatic Coma, Arch. Int. Med. **97**: 762, 1956.
30. Fazekas, J. F., Ticktin, H. E., Ehrmantrout, W. R., and Alman, R. W.: Cerebral Metabolism in Hepatic Insufficiency, Am. J. Med. **21**: 843, 1956.
31. Wilson, W. P., Tyor, M. P., and Kamin, H.: Unpublished observations.

THE HEMOLYTIC EFFECT OF PRIMAQUINE

VIII. THE EFFECT OF DRUG ADMINISTRATION ON PARAMETERS OF PRIMAQUINE SENSITIVITY

C. L. Flanagan, M.D.,* Stanley L. Schrier, M.D.,** Paul E. Carson, M.D.,*** and Alf S. Alving, M.D.****
Chicago, Ill.

AN INTRINSIC defect in erythrocytes has been demonstrated in some Negro Americans and in rare Caucasians. This results in acute intravascular hemolysis when they are given 30 mg. of primaquine base per day or a sufficient dose of similar drugs.[1, 2] Red blood cells from primaquine-sensitive individuals differ from normal erythocytes in at least 4 ways: (1) Their reduced glutathione (GSH) content is lower.[3]† (2) Their GSH is more rapidly destroyed during incubation with acetylphenylhydrazine.[4] (3) They exhibit increased vulnerability to destruction by (a) Heinz body formation in vivo during the first 5 days of drug administration,[5] and, (b) Increased Heinz body formation in vitro when incubated with acetylphenylhydrazine.[6] (4) They are deficient in glucose-6-phosphate dehydrogenase, genetically determined.[8]

These 4 parameters characterize part of the intrinsic red blood cell defect and serve to predict those who will be sensitive to administration of primaquine or related drugs.

Hemolysis is self-limited because only the older cells are destroyed.[9, 10] The remaining red blood cells are resistant to lysis even if administration of 30 mg. primaquine base is continued daily. Therefore, these parameters have been followed during drug-induced hemolysis and recovery to determine if changes in them might point to the site of drug action or to the mechanisms of hemolysis and resistance.

MATERIALS AND METHODS

Subjects were healthy male inmate volunteers of the Illinois State Penitentiary (Stateville). "Sensitive" individuals were defined as those who undergo acute hemolysis when given a standard test dose of 30 mg. primaquine base daily for 14 days.

From the University of Chicago Army Malaria Research Unit, Chicago, Ill.

This work was supported (in major part) by the Research and Development Division, Office of the Surgeon General, Department of the Army, under contract No. DA-49-007-MD-566 with the Department of Medicine, The University of Chicago, Chicago, Ill. Additional support was obtained from Winthrop Laboratories, Inc., New York, N. Y., and the Douglas Smith Foundation, The University of Chicago, Chicago, Ill.

The investigations reported in this paper would not have been possible except for the cooperation of the inmates and the administrative staff of Stateville Penitentiary, Joliet, Ill.

Received for Publication Nov. 8, 1957.

*Formerly Assistant in Medicine, The University of Chicago. Present address: Department of Medicine, Nothwestern University School of Medicine, Chicago, Ill.

**S. A. Surgeon (R), U. S. Public Health Service.

***Surgeon (R), U. S. Public Health Service.

****Department of Medicine, The University of Chicago, Chicago, Ill.

†Normal range in our laboratory is from 58 to 95 mg./100 ml. of red blood cells.

The Glutathione Content of Erythrocytes.—

Determination of reduced glutathione (GSH): Reduced glutathione was determined on freshly drawn heparinized venous blood by the photometric method of Grunert and Phillips.[11] Their procedure depends on the nitroprusside reaction with free sulfhydryl groups. The method is virtually specific for GSH in erythrocytes because metaphosphoric acid filtrates of whole blood contain insignificant amounts of other free sulfhydryl compounds and plasma contains no GSH.[12]

The method of Grunert and Phillips was employed without alteration of the final concentrations of reactants, but certain precautions, not previously described, were found essential in order to obtain accurate and reproducible results.

1. Aqueous solutions of commercial GSH did not give reproducible standard curves* unless they were reduced electrolytically just prior to use.

2. Reagent grade metaphosphoric acid (Fisher) was available as glacial pellets containing 34 to 36 per cent HPO_3, and 58 to 62 per cent $NaPO_3$. Four and a half Gm. of pellets were dissolved in 100 ml. of water giving a solution which was 4.5 per cent metaphosphate, but 1.6 per cent metaphosphoric acid. This was not stated in Grunert and Phillips' paper, nor in previous communications from this laboratory, though this same product has been used in all our determinations.

3. The temperature of the laboratory was maintained between 20° and 22° C. with air conditioning equipment.

4. In a 50 ml. Erlenmeyer flask, 3.0 ml. of whole blood was added to 11.0 ml. of H_2O, mixed, and allowed to stand for 10 to 15 minutes until hemolysis was complete. Then 10.0 ml. of 4.5 per cent metaphosphate solution was added with constant swirling to make an even precipitate; gentle swirling was continued for 5 minutes. From a previously prepared packet, 9.5 Gm. of NaCl was immediately added to the mixture which was shaken vigorously for exactly 2 minutes. This mixture was then filtered through No. 2 Whatman filter paper.†

Beckman cuvettes, 25 mm. in diameter, containing 6 ml. aqueous saturated NaCl were set in advance into a water bath at 20° C. The sodium carbonate (1.5 M)-sodium cyanide (0.067 M) solution and the 2 per cent sodium nitroprusside solution (in an amber bottle) were also equilibrated to 20° C. in the same water bath.

For the determination, 2 ml. of filtrate was delivered into a cuvette, mixed by swirling, and returned to the water bath.‡ Then 1.0 ml. of nitroprusside solution was layered on top by touching the tip of the pipette to the side of the cuvette and letting the solution drain freely down the side. Immediately, 1.0 ml. of cyanide-carbonate solution was added in exactly the same way. The contents were completely mixed by swirling while the outside of the cuvette was dried with a soft cloth. The cuvette was placed in the spectrophotometer and the optical density at 520 mμ was read within 30 seconds after addition of the cyanide-carbonate solution. A fresh blank was prepared for each group of 8 to 12 GSH determinations by adding 2.0 ml. of 2 per cent metaphosphate solution saturated with NaCl to 6.0 ml. of aqueous saturated NaCl. Delivery of nitroprusside and cyanide-carbonate reagents were made as above.§ All filtrates were read in triplicate and agreement to within 3 per cent was obtained on duplicate samples of blood carried through the entire procedure. The pH of the reaction mixture immediately after reading was 9.6 (20° C.).

*Using cuvettes of 25 mm. diameter, the standard curve obtained in a Beckman Model B spectrophotometer had about 1.5 times the slope of the curve obtained in the Coleman Junior spectrophotometer.

†The amounts given above were used for determinations of both reduced and total glutathione. For determination of GSH only, the amounts were halved.

‡The directions are for the flat-bottomed cuvettes. For Coleman cuvettes a stirring rod was used.

§The concentration of the cyanide-carbonate and nitroprusside solutions can be controlled by reading the blank against distilled H_2O at 520 mμ. In the Beckman B spectrophotometer the O.D. should be 0.100 ± 0.002.

5. Standard curves were checked each time new reagents were made. A standard solution of reduced glutathione, 40 mg./100 ml. of water, was prepared weekly and kept refrigerated. An aliquot of this standard solution was electrolytically reduced and run with each batch of glutathione determinations.

Determination of total and oxidized blood glutathione (GSSG): Total glutathione content of blood was also determined by the method of Grunert and Phillips after electrolytic reduction of metaphosphoric acid filtrates according to our adaptation of the method of Dohan and Woodward.[13] After electrolytic reduction, blood plasma contained 13 ± 3 μm of sulfhydryl per 100 ml. of plasma. The amount of sulfhydryl in the plasma was very constant and was accounted for by the disulfide amino acids consisting almost entirely of cystine. Correction was made for disulfide in plasma in calculating the total glutathione content of the red cells.

1. Ten milliliters of metaphosphoric acid filtrates of blood obtained as described above were reduced over a mercury cathode in a 100 ml. beaker (diameter 4.5 cm.) at 36 Ma. (50 volts) for 15 minutes at a current density of 2.2 Ma./cm.^{2} The pH of the filtrate was 1.0 before reduction and 5.6 after.

2. A 4 per cent solution of sulfosalicylic acid was used at the anode.

3. It was absolutely essential to "reduce" the triple distilled mercury cathode for at least 30 minutes immediately prior to each reduction of filtrate by immersion of the agar bridge into the mercury at the same current density.

4. The mercury cathode had to be washed many times with distilled water and blotted dry after each reduction of filtrate. The mercury surface was covered with distilled water when not in use.

5. A saturated 4 per cent KCl agar bridge in 6 to 8 mm. diameter glass tubing was used. Frequent examination of the glass-agar junction was necessary because hydrogen bubbles tended to accumulate and creep between the glass and the agar, leading to low results. It was then necessary to cut off about 5 mm. of glass tubing and allow a fresh portion of agar to protrude.

6. After reduction, total glutathione was determined on whole blood and plasma by the method of Grunert and Phillips as described above. Duplicate hematocrits were done on each sample of blood in order to calculate results in mg. of glutathione per 100 ml. of red blood cells thereby eliminating differences introduced by hemodilution, hemoconcentration, or anemia. Oxidized glutathione of the erythrocytes was estimated by subtracting the reduced glutathione from the total glutathione.*

In Vitro Heinz Body Formation.—Determination of in vitro Heinz body formation was performed as previously described.[6] In addition "a modified test procedure" was devised to compensate for variations in hematocrit. In the latter procedure, 5 ml. of freshly drawn venous blood was placed in a Cutler blood sedimentation tube and centrifuged for 3 to 5 minutes. Sufficient plasma was withdrawn to correct the hematocrit to approximately 50 per cent. The sedimented blood was immediately resuspended, and the standard test procedure was carried out.

Survival of Erythrocytes.—Survival curves of red blood cells labeled with Cr^{51} were performed as described previously.[1]

Glucose-6-Phosphate Dehydrogenase.—Enzyme assays of glucose-6-phosphate dehydrogenase were carried out on hemolyzates by a modification of the procedures described by Carson and associates.[7]†

Glutathione Stability.—The glutathione stability test was performed as described by Beutler.[4]

*Oxidized glutathione obtained from Schwarz Laboratories and analyzed by this technique gave recovery of between 83 and 87 per cent of weighed quantities. This recovery was entirely in agreement with recoveries of the same material obtained by amperometric titration, chromatography, and elemental analysis for nitrogen and sulfur. The remaining material was said to consist of "waters and alcohols of hydration."[14]

†According to the original publication in *Science* **124**: 484, 1956, hemolyzates were dialyzed overnight against 0.67 M phosphate buffer. This was an error. Dialysis was performed against 0.067 M phosphate buffer.

RESULTS

Effect of Drug Administration on Reduced and Total Glutathione Content of Red Blood Cells.—

Normal individuals: Reduced and total glutathione were determined every other day for 8 days on blood obtained from each subject at 8 A.M. Thirty milligrams of primaquine base were then given as a single daily dose to each subject and blood was drawn daily at 8 A.M. for determination of reduced and total glutathione content.* No significant change in either reduced or oxidized glutathione was found (Fig. 1). There was no evidence of hemolysis either by shortened survival of Cr^{51}-tagged red blood cells or by change in hemogram.

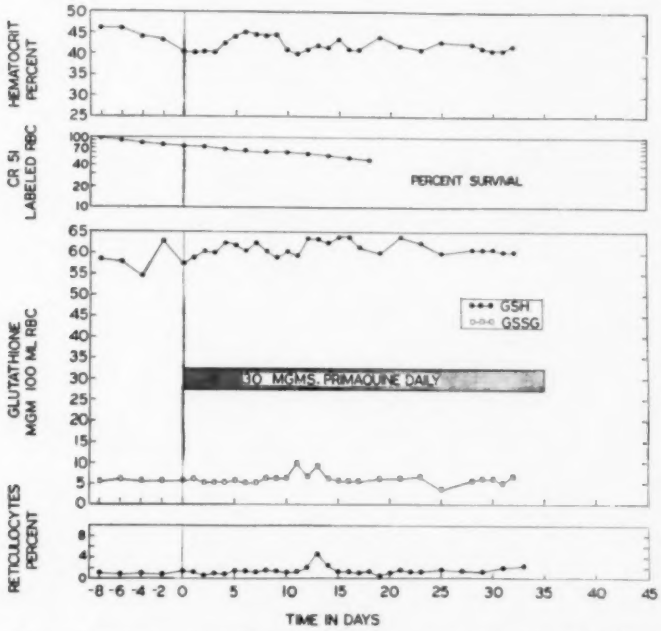


Fig. 1.—Effect of primaquine administration on a nonsensitive individual.

Primaquine sensitive individuals: The effect of 30 mg. of primaquine base administered to sensitive individuals daily is illustrated in Fig. 2 which represents the composite findings of 3 individuals. During the first 3 days of drug administration, the reduced glutathione fell sharply with only a slight fall in the hematocrit and the slope of the Cr^{51} curve. The GSH then rose rapidly during the period of major hemolysis which lasted at least through day 12, as evidenced by a sharp fall in the hematocrit and the destruction of Cr^{51}-labeled erythrocytes. Increased erythropoesis began on the fourth or fifth day

*During some experiments reduced and oxidized glutathione were determined on blood drawn every 2 to 4 hours from both normal and primaquine sensitive individuals. The results were in no way different from those described above.

and reached a peak on day 9, before the GSH had returned to its pretreatment level. The GSH continued to rise gradually after the reticulocytes in the blood returned to normal values. The imperfect correlation between the reticulocyte response and the rise in erythrocyte GSH content to pretreatment levels indicated that the outpouring of young erythrocytes was not the only factor involved. The oxidized glutathione content of the sensitive erythrocytes, which prior to hemolysis was the same as that found in normal erythrocytes, was unaffected during hemolysis and subsequent recovery.*

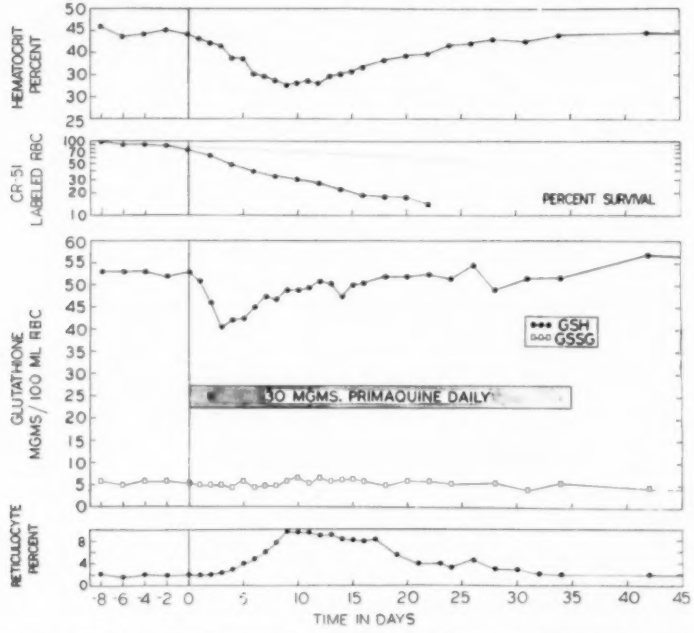


Fig. 2.—Composite graph showing the effect of primaquine administration on 3 sensitive individuals.

The above sequence of events has been observed consistently and indicates the following conclusions. (1) The reduced glutathione of the erythrocytes was destroyed *before* the removal of the majority of sensitive cells from the circulation. (2) The return of reduced glutathione toward pretreatment values coincided with the major destruction of susceptible older cells, in which, presumably, the reduced glutathione had previously been destroyed. (3) Drug destroyed only the reduced glutathione of the erythrocytes and did not measurably affect oxidized glutathione, which remained normal in erythrocytes of both normal and drug-sensitive subjects during drug administration.

*Recently, it was reported that $GSSG^{35}$ content (determined by iodometric titration) was significantly higher in normal erythrocytes than in drug sensitive erythrocytes. Using the more specific and sensitive nitroprusside method and correcting for the appreciable factor of plasma disulfides, we found that the GSSG content of normal and drug-sensitive erythrocytes was the same.

Effect of sulfanilamide and acetanilide: The daily administration of 3.6 Gm. sulfanilamide and 3.6 Gm. of acetanilide to primaquine-sensitive individuals induced an identical course of events.*

The Effect of Drug Administration on the Glutathione Stability Test.— The glutathione stability test[4] was performed on normal and sensitive individuals before, during, and after a course of 30 mg. of primaquine base daily. In neither normal nor sensitive individuals was there a significant change (Table I).

TABLE I. GLUTATHIONE STABILITY TEST

SUBJECTS	BEFORE DRUG ADMINISTRATION	DURING DRUG ADMINISTRATION	AFTER DRUG ADMINISTRATION
Rob.*	18.2	21.5	23.8
Jac.*	6.8	26.4	11.6
McC.*	7.8	10.5	6.7
Wri.*	11.5	14.3	16.9
Per.†	65.0	60.1	54.4
Byb.†	60.3	74.5	68.3
Gra.†	57.8	74.1	54.7

Results are expressed in mg. GSH/100 ml. R.B.C. remaining after 2 hours' incubation at 37° C. with 5 mg. acetylphenylhydrazine/ml. blood.

In our laboratory primaquine-sensitive individuals have a fall in GSH to below 30 mg./100 ml. red blood cells.

*Primaquine-sensitive subject.

†Nonsensitive subject.

TABLE II. THE VARIATION IN RESPONSE OF A SENSITIVE SUBJECT TO THE IN VITRO FORMATION OF HEINZ BODIES BEFORE, DURING, AND AFTER HEMOLYSIS

DATE	STANDARD TEST			MODIFIED TEST
	DRUG ADMINISTRATION	HEMATOCRIT	H-B* (PER CENT)	H-B* (PER CENT)
8/18/55		44	54	38
8/20/55		44	43	35
8/22/55		43	66	43
8/24/55		43	60	42
8/26/55		43	77	61
8/28/55	Primaquine	42	81	66
8/30/55	30 mg. base	37	72	62
9/ 1/55	per day	34	76	63
9/ 3/55		34	69	50
9/ 5/55		36	77	57
9/ 7/55		38	73	51
9/ 9/55		41	66	54
9/11/55		44	68	59
9/13/55		44	43	54
9/15/55		44	66	61

*The percentage of cells containing 5 or more Heinz bodies. Normal individuals form less than 30 per cent Heinz bodies under these conditions.

Effect of Acute Drug-Induced Hemolysis on Heinz Body Formation In Vitro.—Table II illustrates the effect of the acute hemolytic episode on the susceptibility of red blood cells to Heinz body formation in vitro in the sensitive

*Phenylhydrazine in large doses (90 mg. daily) uniformly produced an anemia in normal subjects which differed in some ways from primaquine-induced anemia in sensitive subjects. In a normal individual the glutathione did not fall sharply in the first 3 days of phenylhydrazine administration, but instead fell very slowly and continued to fall for as long as 2½ months after drug was discontinued. The major hemolysis actually preceded, rather than followed, the fall in glutathione. In a normal individual there was a profound and continuing anemia without the "escape" seen with compounds that act like primaquine.[16]

individual. During drug administration the test results increased slightly by both the standard and modified procedures. However, it was significant that during the recovery phase, at a time when the cells were no longer sensitive to administration of 30 mg. of primaquine base in vivo, and no longer formed Heinz bodies in vivo, the in vitro test results remained high.

Effect of Drug Administration on the Glucose-6-Phosphate Dehydrogenase.—In hemolyzates from normal individuals there was no significant change in glucose-6-phosphate dehydrogenase during drug administration. During the course of hemolysis in sensitive individuals there was a slight rise in glucose-6-phosphate dehydrogenase which seemed to parallel the reticulocytosis. The glucose-6-phosphate dehydrogenase never approached normal values and returned to low pretreatment levels within one month, whether or not drug administration was continued.

One drug-sensitive individual has been treated with 30 mg. of primaquine base daily for 1 year after undergoing a primaquine-induced hemolysis. The glucose-6-phosphate dehydrogenase returned to pretreatment value shortly after the acute episode and has since remained constant. His hemogram and Cr^{51} curve have been normal since recovery from the initial hemolysis.

DISCUSSION

Our data indicate that in primaquine-sensitive individuals a phase of acute reduced glutathione destruction precedes the major hemolysis. The results are consistent with the interpretation that the reduced glutathione of the older sensitive erythrocytes is destroyed in the first phase, after which the cells hemolyze. This observation emphasizes the significance of erythrocyte glutathione for the maintenance of cell integrity.[17, 18]

During the first 3 days of drug administration, 300 mg. or more of reduced glutathione may disappear from erythrocytes (Fig. 2), and yet their oxidized glutathione content remains constant. This fact does not support Beutler's assumption[19] that these hemolytic drugs cause oxidation of GSH to GSSG in vivo, as they do in vitro. Reduced glutathione may be destroyed by hydrolysis to its component amino acids or dipeptides, since in vivo drug administration does not affect oxidized glutathione and does not apparently oxidize reduced glutathione of the erythrocytes.

The fact that Heinz body production, the stability of reduced glutathione tested in vitro, and glucose-6-phosphate dehydrogenase of sensitive red cells do not become normal during recovery, indicates that our knowledge of the mechanism of in vivo hemolysis is incomplete. The slight transient rise in glucose-6-phosphate dehydrogenase occurring during increased erythropoiesis is adequately explained by Marks's[20] observations that young erythrocytes have more glucose-6-phosphate dehydrogenase than do older cells. The important consideration, however, is that even the young, resistant cells of sensitive subjects have much less enzyme than do cells of normal subjects. Our current study is concerned with glutathione synthesis and destruction as well as the parts played by glucose-6-phosphate dehydrogenase, glutathione reductase,[21] and coenzymes in the hemolytic reaction and recovery.

SUMMARY

1. Administration of primaquine to normal individuals caused no significant change in the reduced (GSH) and oxidized (GSSG) glutathione content of their erythrocytes. Similar drug administration to primaquine-sensitive individuals resulted in a sharp fall of reduced glutathione followed by acute hemolysis which occurred in the face of rising glutathione values.

2. The effect of sulfanilamide and acetanilide was entirely similar to that of primaquine.

3. The GSSG content of erythrocytes of primaquine-sensitive individuals was normal and was not altered during hemolysis induced by 30 mg. of primaquine base administered daily. These data do not support the assumption, based on in vitro observations, that the fall in GSH content of the erythrocytes of sensitive subjects results from conversion of GSH to GSSG during hemolysis.

4. After hemolysis the young and newly formed erythrocytes remaining in the circulation were resistant to further hemolysis when 30 mg. of primaquine base was continued daily for one year. However, the resistant cells still had low glutathione content, low in vitro glutathione stability, increased Heinz body formation in vitro, and were deficient in glucose-6-phosphate dehydrogenase. The fact that these parameters did not become normal after recovery even though the red blood cells had become refractory to further hemolysis with 30 mg. primaquine base indicates that our knowledge of the factors contributing to hemolysis is incomplete.

REFERENCES

1. Dern, R. J., Weinstein, I. M., LeRoy, G. V., Talmage, D. W., and Alving, Alf S.: The Hemolytic Effect of Primaquine. I. The Localization of the Drug-Induced Hemolytic Defect in Primaquine-Sensitive Individuals, J. LAB. & CLIN. MED. **43**: 303-309, 1954.
2. Dern, R. J., Beutler, E., and Alving, Alf S.: The Hemolytic Effect of Primaquine. V. Primaquine-Sensitivity as a Manifestation of a Multiple Drug-Sensitivity, J. LAB. & CLIN. MED. **45**: 30-39, 1955.
3. Beutler, E., Dern, R. J., Flanagan, C. L., and Alving, Alf S.: The Hemolytic Effect of Primaquine. VII. Biochemical Studies of Drug-Sensitive Erythrocytes, J. LAB. & CLIN. MED. **45**: 286-295, 1955.
4. Beutler, E.: The Glutathione Instability of Drug-Sensitive Red Cells, J. LAB. & CLIN. MED. **49**: 84-95, 1957.
5. Beutler, E., Dern, R. J., and Alving, Alf S.: The Hemolytic Effect of Primaquine. III. A Study of Primaquine-Sensitive Erythrocytes, J. LAB. & CLIN. MED. **44**: 177-184, 1954.
6. Beutler, E., Dern, R. J., and Alving, Alf S.: The Hemolytic Effect of Primaquine. VI. An In Vitro Test for Sensitivity of Erythrocytes of Primaquine, J. LAB. & CLIN. MED. **45**: 40-50, 1955.
7. Carson, P. E., Flanagan, C. L., Ickes, C. E., and Alving, A. S.: Enzymatic Deficiency in Primaquine-Sensitive Erythrocytes, Science **124**: 484-485, 1956.
8. Tarlov, A. R., Kellermeyer, R. W., Schrier, S. L., Carson, P. E., and Alving, A. S.: Unpublished data.
9. Beutler, E., Dern, R. J., and Alving, Alf S.: The Hemolytic Effect of Primaquine. IV. The Relationship of Cell Age to Hemolysis, J. LAB. & CLIN. MED. **44**: 439-442, 1954.
10. Dern, R. J., Beutler, E., and Alving, Alf S.: The Hemolytic Effect of Primaquine. II. The Natural Course of the Hemolytic Anemia and the Mechanism of Its Self-Limited Character, J. LAB. & CLIN. MED. **44**: 171-176, 1954.
11. Grunert, R. R., and Phillips, P. H.: A Modification of the Nitroprusside Method of Analysis for Glutathione, Arch. Biochem. **30**: 217-225, 1951.
12. Behrendt, H.: Chemistry of Erythrocytes, Springfield, Ill., 1957, Charles C Thomas, pp. 57, 59, 60, and 64.

13. Dohan, J. S., and Woodward, G. E.: Electrolytic Reduction and Determination of Oxidized Glutathione, J. Biol. Chem. **129:** 393-403, 1939.
14. Schwarz Laboratories. Personal communication.
15. Szeinberg, A., and Chari-Bitron, A.: Blood Glutathione Concentration After Haemolytic Anaemia Due to Vicia Faba or Sulphonamides, Acta Haemat. **18:** 229-233, 1957.
16. Flanagan, C. L.: Unpublished observations.
17. Fegler, G.: Relationship Between Reduced Glutathione Content and Spontaneous Hemolysis in Shed Blood, Nature **170:** 624-625, 1952.
18. Sheets, R. F., Hamilton, H. E., and DeGarvin, E. L.: Hemolysis of Human Erythrocytes by a Sulfhydryl Inhibitor, p-Chloromercuribenzoic Acid, Proc. Soc. Exper. Biol. & Med. **91:** 423-427, 1956.
19. Beutler, E., Robson, M., and Buttenwieser, E.: The Mechanism of Glutathione Destruction and Protection in Drug-Sensitive and Non-Sensitive Erythrocytes. In Vitro Studies, J. Clin. Invest. **36:** 617-628, 1957.
20. Marks, P. A.: A Relationship Between Erythrocyte Aging In Vivo and Activities of Glucose-6-Phosphate and 6-Phosphogluconic Dehydrogenase, J. Clin. Invest. **36:** 913, 1957.
21. Schrier, S. L., Kellermeyer, R. W., Carson, P. E., and Alving, Alf S.: A Second Enzyme Abnormality in Primaquine Sensitive Erythrocytes, J. LAB. & CLIN. MED. (Abst.) **50:** 951, 1957.

LABORATORY METHODS

DETERMINATION OF BROMIDE SPACE BY MEANS OF A POLAROGRAPHIC METHOD

HELEN S. JONES, PH.D., AND MARY L. TERRY, A.B.
BOSTON, MASS.

EXTRACELLULAR volume often constitutes an important variable in studies of the effect of changing conditions upon the water and electrolyte composition of the body. As Brodie,[2, 3] Gamble,[4] and Cheek[5, 6] have shown, extracellular volume can be estimated satisfactorily by measuring the dilution of a tracer quantity of bromide (less than 10 mEq. per liter of serum). Brodie's and Gamble's measurements were dependent upon the oxidation of bromide to bromate[1] while Cheek utilized the microdiffusion technique of Conway.[7] Though these techniques are reproducible, they both present certain technical difficulties. The present study was undertaken in an effort to find a simple polarographic method for the analysis of bromide in microsamples of serum.

The problem with respect to polarographic estimation of bromide in serum is that the half-wave potential for the anodic oxidation of bromide ion at the dropping mercury electrode is only approximately 0.13 volt more negative than that of chloride ion, the concentration of which in serum is very large relative to any concentration of bromide which would be used in space measurements. Nevertheless, using a comparative method of polarographic analysis[8] and measuring diffusion currents at –0.230 v. versus the mercurous sulfate half-cell (approximately the half-wave potential for oxidation of bromide ion in phosphotungstic acid), it has been possible to demonstrate a linear relationship between diffusion current and bromide concentration which is not affected by changes in chloride concentration in a range corresponding to 80 to 120 mEq. per liter of serum.

MATERIALS AND METHODS

Apparatus.—Patwin electro-polarizer, Model R1. The electro-polarizer is used at full sensitivity, giving 0.00446 microampere per millimeter on the galvanometer scale.

A dropping mercury electrode, delivering 1.5 mg. Hg per second is used. The drop time at –0.230 volt in the solutions described is 3.4 seconds and is kept constant during any

From the Department of Pediatrics, Harvard Medical School, and the Children's Medical Service, Massachusetts General Hospital, Boston, Mass.

This work was supported by grants from The Commonwealth Fund of New York and Grant H-1529 of The National Heart Institute, United States Public Health Service under the direction of N. B. Talbot, M.D.

Received for publication July 1, 1957.

series of readings. A convenient assembly consists of 8.5 cm. of capillary tubing, inside diameter 0.051 mm., outside diameter 5 mm., with the height of the mercury column above the capillary maintained at about 73 cm. by a leveling bulb.

Mercurous sulfate half-cell.[9]

Five-milliliter beakers (Fisher No. 11-505-155).

Temperature controlled room or constant temperature water bath. The procedure is satisfactorily performed at any constant temperature in the range from 22° to 27° C.

Reagents.—Twelve gram per cent sodium tungstate solution.

Two and four-tenths M phosphoric acid [160 ml. of phosphoric acid (H_3PO_4, sp. gr. 1.7) per liter].

Stock standard potassium bromide, 10 mEq. per liter (1.19 Gm. of pure, dry, reagent grade potassium bromide per liter). Aliquots of this solution are diluted 1:20 and 1:50 to yield standard solutions containing 0.5 and 0.2 mEq. of bromide per liter, respectively.

Procedure.—To measure bromide in serum, it is necessary to obtain 0.5 ml. of serum from the subject (control serum) prior to bromide administration. The determination of serum bromide concentration following bromide administration can then be accomplished in duplicate with a 0.3 ml. serum sample.

The basic supporting electrolyte solution used throughout contains 1.0 ml. of 12 per cent sodium tungstate and 1.0 ml. of 2.4 M phosphoric acid per 10 ml. of water solution.

Two standard solutions containing known amounts of bromide in serum are prepared by adding 1.0 ml. of the 0.2 and 0.5 mEq. per liter standard bromide solutions, respectively, to 0.1 ml. aliquots of the control serum in 10 ml. glass-stoppered, graduated mixing cylinders. To each is added 1.0 ml. of sodium tungstate. Thereafter the volume is made up to 9.0 ml. with water and finally to 10 ml. by the addition of 1.0 ml. of phosphoric acid. The unknowns are treated similarly except that no bromide is included. The solutions are thoroughly mixed, transferred to clean dry tubes, allowed to stand for 10 to 15 minutes until the protein starts to settle and centrifuged for 5 minutes at about 3,000 r.p.m. The clear supernatant solutions are poured into clean, dry rubber-stoppered tubes.

The diffusion currents of these solutions may be measured as soon as all have reached constant temperature or they may be allowed to stand over a period of days. However, solutions prepared simultaneously should be read as a group. Just prior to measurement, approximately 4.5 ml. of the solution is transferred to a 5 ml. Fisher beaker. Oxygen need not be removed from the solutions. The capillary tip of the dropping mercury electrode and the tip of the agar bridge from the reference electrode are aligned to dip into the solution in the beaker about halfway between the surface and the bottom so that beakers can be changed easily without disturbing the alignment. The diffusion current of each solution is measured at a fixed applied voltage of −0.230. The comparative polarographic procedure obviates the need for measurement of residual current. Each measurement is made in duplicate and the average of the two readings is used.

The concentrations of bromide in the standard solutions are plotted against the galvanometer readings, and the bromide concentration of the unknown is interpolated directly from this line. This value multiplied by the dilution factor (100) gives the mEq. of bromide per liter of serum.

EXPERIMENTAL AND DISCUSSION

Sulfuric acid (0.1 N), potassium nitrate (0.1 N and 1.0 N), and the Somogyi zinc sulfate—sodium hydroxide precipitant,[10] as well as phosphotungstic acid, were investigated as possibilities for the supporting electrolyte. The

best defined waves and the most consistent results were obtained when phosphotungstic acid in the proportions specified above was used. The solution also served to precipitate serum proteins.

Fig. 1 shows the current-voltage relationships observed in solutions of supporting electrolyte alone (SE).; supporting electrolyte containing 1.0 mEq. of chloride per liter (Cl^-); supporting electrolyte containing 0.05 mEq. of bromide per liter (Br^-); and supporting electrolyte containing both 1 mEq. of chloride and 0.05 mEq. of bromide per liter ($Cl^- + Br^-$). It is evident that there is no sharp rise in current due to chloride until the applied voltage reaches –0.200 versus the mercurous sulfate electrode. The diffusion current for bromide, on the other hand, begins to rise at a distinctly more negative voltage. This suggests that bromide might be measured in the presence of chloride at an applied voltage between –0.260 and –0.210. The relations observed when the two ions are present together in the above concentrations indicate that at –0.230 v. the current contribution due to chloride is very small.

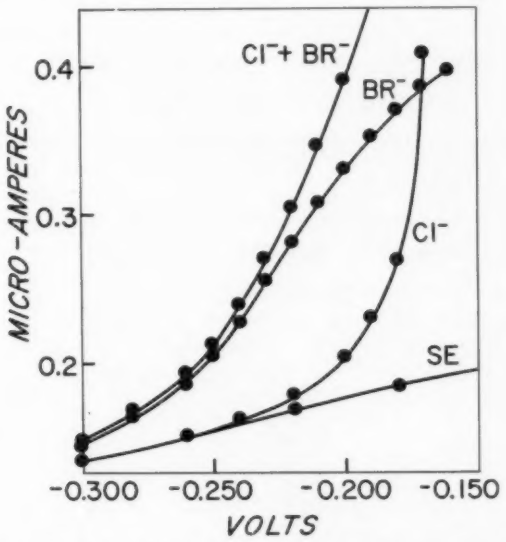


Fig. 1.—A comparison of the current-voltage curves for Cl^-, Br^-, and $Cl^- + Br^-$ in phosphotungstic acid (S.E.). T = 24.2° C., $[Cl^-]$ = 1.0 mEq./L. $[Br^-]$ = 0.05 mEq./L.

Using –0.230 v. as applied voltage, a study of the relationship between diffusion current and bromide concentration was made. A linear relationship was found between current and concentration ranging from 0.02 to 0.1 mEq. of bromide per liter in the presence of 1.0 mEq. of chloride per liter (Fig. 2, broken line). Equivalent bromide concentrations in the presence of serum (0.1 ml. per 10 ml. of supporting electrolyte, giving approximately 1.0 mEq. of chloride per liter) were studied at the same time. Linearity pertained, but the rate of increase of diffusion current was greater in the presence of serum (Fig. 2, solid line). At zero bromide concentration, current readings may be above

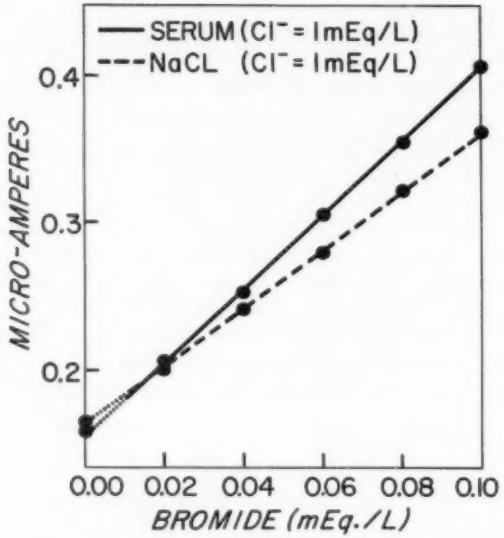


Fig. 2.—A comparison of the diffusion current-bromide concentration curves in solutions prepared with serum and with NaCl. All solutions were prepared at the same time. T = 23.7° C.

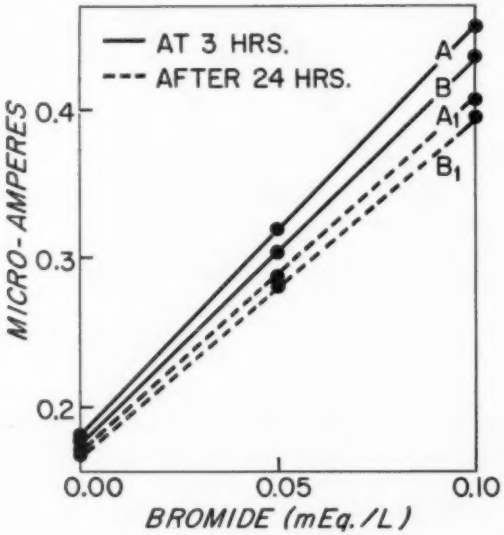


Fig. 3.—A comparison of the diffusion current-bromide concentration curves for 2 different sera. All solutions were prepared at the same time and measurements were made both at 3 and 24 hours. T = 24.5° C.

the extrapolation of the straight line which has been found to pertain for bromide concentrations between 0.01 and 0.10 mEq. per liter. Therefore greatest accuracy is achieved by measuring an unknown solution between two standards containing bromide.

Calibration curves of the type shown in Fig. 2 have been obtained with equivalent amounts of potassium bromide in the presence of 0.1 ml. aliquots of various sera. The concentration-diffusion current relation in each instance is linear, but, as shown by Fig. 3, lines A and B, the origins and slopes of the lines are not the same for all specimens. It also has been found that the origins and slopes shift if the solutions are allowed to stand for long periods before measurements of the diffusion currents are made (compare solid lines A and B with broken lines A_1 and B_1). On the basis of these findings it is essential that each unknown be determined with reference to standards prepared by the addition of known amounts of bromide to the individual's control serum and that the solutions to be used for calibration and for measurement be prepared simultaneously and read simultaneously.

The effect of changes in chloride concentration have been studied as follows. The chloride concentration of aliquots of a serum containing 80 mEq. of chloride per liter was increased to 100 and 120 mEq. per liter by the addition of sodium chloride. As shown by Table I, at –0.230 v. these changes in chloride concentration were without appreciable influence on the diffusion currents produced by 5 and 10 mEq. of bromide per liter of serum. Thus, as would be predicted from Fig. 1, variations in chloride concentration per se of the small order of magnitude which might be encountered in sera drawn from the same patient at a 3 to 4 hour interval, do not give rise to important errors in serum bromide measurements.

TABLE I. EFFECT OF CHANGES IN CHLORIDE CONCENTRATION ON MEASUREMENT OF BROMIDE

BROMIDE CONC. (MEQ./L.)	CHLORIDE CONC. (MEQ./L.)	MICROAMPERES AT –0.230 V.	APPARENT CHANGE IN Br^- CONC. DUE TO CHLORIDE (MEQ./L.)
0.0	0.8	0.161	----
0.0	1.0	0.163	0.001
0.0	1.2	0.165	0.002
0.05	0.8	0.278	----
0.05	1.0	0.285	0.003
0.05	1.2	0.285	0.003
0.1	0.8	0.403	----
0.1	1.0	0.408	0.002
0.1	1.2	0.410	0.003

It was found, however, that if the amount of serum added to 10 ml. of supporting electrolyte is doubled, 1.5 ml. of the sodium tungstate must be used to maintain the linear relationship between diffusion current and bromide concentration. In such a system the sensitivity is further increased (see Fig. 2) but a change in serum chloride concentration of as little as 5 mEq. per liter is sufficient to destroy the linear relationship if the bromide concentration in serum is above 2.5 mEq. per liter.

Error.—To determine the error of the procedure, calibration curves were made with 5 different sera. Bromide was added to separate 0.1 ml. aliquots of

each serum to give concentrations of 0.01, 0.02, 0.03, 0.04, and 0.05 mEq. per liter in supporting electrolyte. The diffusion currents at –0.230 v. were measured. In each instance the average slope of the line defined by these measurements was calculated and the deviation of the individual points from this average line determined. The results obtained revealed the maximum error to be equivalent to ±0.05 mEq. of bromide per liter of serum, and the standard deviation of individual determinations to be ±0.023 mEq. per liter. In a series of 11 duplicate determinations on sera having a range of bromide concentration from 1.3 to 3.8 mEq. per liter, the standard deviation computed from the means of the pairs was ±0.04 mEq. per liter.

TABLE II. POLAROGRAPHIC MEASUREMENT OF BROMIDE SPACE IN INDIVIDUALS OF VARYING SIZE

CASE NO.	DIAGNOSIS	STATE OF HYDRATION	BODY WEIGHT (KG.)	BROMIDE ADMINISTERED (MEQ/KG.)		SERUM BROMIDE (MEQ./L.)	BROMIDE SPACE (L.)	BROMIDE SPACE (PER CENT BODY WT.)
1.	Normal man	Normal	75.0	0.78	P.O.	2.60	17.8	23.7
2.	Normal woman	Normal	63.7	0.79	P.O.	2.85	13.9	21.8
3.	Uveomeningitis (Harada's syndrome)	Normal	40.0	0.76	P.O.	2.82	8.30	20.8
4.	Ulcerative colitis	Normal	38.0	0.89	I.V.	2.92	9.15	24.0
5.	Cystine storage disease	Normal	15.0	0.88	P.O.	3.00	3.39	22.6
6.	Anorexia nervosa	Pitting edema	24.8	0.70	P.O.	1.45	9.45	38.1
7.	Intestinal atresia	Pitting edema	11.0	0.97	P.O.	2.10	4.03	36.6
8.	Malrotation of bowel	Water intoxication	3.0	0.94	I.V.	1.82	1.25	41.7

RESULTS

Table II presents bromide space data obtained on 8 individuals of various sizes: 5 were normally hydrated, 2 were edematous, and 1 was suffering from postoperative water intoxication. In each instance a known amount of bromide was administered by mouth, or given intravenously. A small volume of 5 per cent dextrose in water was used as a vehicle for intravenous administration and the infusion time kept to one hour or less. Blood samples were obtained just prior to administration and again 3 to 4 hours later, in accordance with the observations of Cheek.[5, 6]

Bromide space was calculated by the following equation:

$$\text{Bromide Space in Liters} = \frac{\text{mEq. Bromide administered}}{\text{Serum Bromide in mEq./L.}} \times 0.9 \div \left(\frac{\text{Serum Bromide in mEq./L.}}{0.95 \times \text{Serum Water in Gm./Gm. serum}}\right).$$

The bromide administered is multiplied by 0.9 to correct for entry of 10 per cent into the red blood cells; 0.95 is the Donnan factor; serum water is determined by weight. No correction was made for the negligible urinary excretion of bromide ion.

The results obtained on both the normally hydrated and the edematous and hyperhydrated subjects are in keeping with the findings of others.[2-6]

The use of comparative polarography with the sensitivity here indicated suggests that this method will be suitable for repetitive determinations of bromide space. When repeated determinations are done at time intervals so short that residual bromide will be found in the serum, this residum should not interfere with the independent measurement of newly introduced bromide.

SUMMARY

A polarographic method for the determination of bromide in 0.1 ml. of serum is described. Under controlled conditions, this permits the measurement of bromide at concentrations of 1.0 to 10.0, s.d. ±0.04, mEq. per liter. The method is discussed and its use for the determination of bromide space is exemplified.

We are indebted to J. D. Crawford, M.D., and N. B. Talbot, M.D., for their generous and invaluable assistance in the preparation of this manuscript. We also wish to thank Patwin Instruments, Waterbury, Connecticut, who very generously provided us with the electro-polarizer on which these measurements were made.

REFERENCES

1. Brodie, B. B., and Friedman, M. M.: The Determination of Bromide in Tissues and Biological Fluids, J. Biol. Chem. **124**: 511-518, 1938.
2. Brodie, B. B., Brand, E., and Leshin, S.: The Use of Bromide as a Measure of Extracellular Fluid, J. Biol. Chem. **130**: 555-563, 1939.
3. Brodie, B. B., Friedman, M. M., and Ferraro, L. R.: Studies on Extra-cellular Fluid, Proc. Am. Soc. Biol. Chem., J. Biol. Chem. **140**, XXI, 1941.
4. Gamble, J. L., Jr., Robertson, J. S., Hannigan, C. A., Foster, C. G., and Farr, L. E.: Chloride, Bromide, Sodium and Sucrose Spaces in Man, J. Clin. Invest. **32**: 483-489, 1953.
5. Cheek, D. B.: Estimation of Bromide Space With a Modification of Conway's Method, J. Appl. Physiol. **5**: 639-645, 1953.
6. Cheek, D. B.: Observations on Total Body Chloride in Children, Pediatrics **14**: 5-10, 1954.
7. Conway, E. J.: Microdiffusion Analysis and Volumetric Error, ed. 2, London, 1950, D. Van Nostrand Company, Inc., p. 196.
8. Meites, L.: Polarographic Techniques, New York, 1955, Interscience Publishers, Inc., p. 175.
9. Kolthoff, I. M., and Lingane, J. J.: Polarography, ed. 2, New York, 1952, Interscience Publishers, Inc., vol. 1, p. 362.
10. Somogyi, M.: A Method for the Preparation of Blood Filtrates for the Determination of Sugar, J. Biol. Chem. **86**: 655-663, 1930.

SERUM PROTEIN PARTITION ANALYSIS: CORRELATION BETWEEN PAPER ELECTROPHORETIC AND TURBIDIMETRIC METHODS

D. R. Davis, B.S., F. A. Teague, B. J. Dietz, and R. E. Budd
Bethesda, Md.

HOWE'S method of serum protein analysis[2] and subsequent modifications were universally used to differentiate between normally and abnormally occurring globulin fractions before the application of electrophoresis into biologic and physical problems, by Tiselius in 1937, and more recently paper electrophoresis.[1] When the results obtained by the Tiselius method of free electrophoresis were compared with those of Howe's method they did not agree, primarily because a portion of the alpha globulins is not precipitated with the other globulins.[3-7] Hans Popper[8] statistically correlated the results of protein partition analysis obtained by both free electrophoresis and Wolfson's salt partition method.[9] Several other salt precipitation methods have been reported.[10-15] However, the results have generally been similar to those obtained by Howe and Popper.

Recently,[17] Aull reported a comparison between a phosphate-turbidity method and Wolfson's sodium sulfate-sulfite method. The authors intend to compare paper electrophoresis and Aull's turbidimetric method, thus hoping to obtain a truer electrophoretic-chemical relationship for serum proteins if such a relationship is feasible.

MATERIALS AND METHODS

Comparative paper electrophoresis and turbidimetric studies were done on 49 healthy adults (normals) and 13 patients chosen because of abnormal serum electrophoretic patterns. All cases were fasting prior to the drawing of blood, and serum was used for analysis. All values were reported as percentage distribution of the whole rather than as grams per cent in order to facilitate comparisons between patients with different total protein values.[23]

Electrophoresis.—Electrophoretic separation and staining of the resultant strips was carried out by the method described by Jencks and associates.[18] The dyed strips were "blued" by exposing them to an ammonium hydroxide atmosphere and scanned with an automatic recording and integrating densitometer.[19]

Protein.—Total protein was determined by the biuret method.[20]

Chemical.—The phosphate-turbidity method described by Aull and McCord[17] was employed.

The electrophoretically determined data for concentration of albumin, albumin + alpha-1, alpha-2, alpha-1+2, beta, and gamma globulins were plotted against comparative findings obtained by the turbidimetric method in the same specimens. The significance of

From the RADM G. W. Calver's Physical Chemistry Research Laboratory, Naval Medical School, National Naval Medical Center, Bethesda 14, Md.
Received for publication July 3, 1957.

the correlation in the resultant plots was tested by Pearson's product-moment correlation coefficient[22]: $r_{xy} = \frac{\Sigma (xy)}{N\sigma_x \sigma_y}$ where x and y are the deviations of the observed values from the respective means, N is the total number of cases, and σ_x and σ_y are the standard deviations of the respective means.

With this calculation, an r of 1.0 represents perfect correlation whereas an r of 0 indicates no correlation. In each instance, the standard error of estimate of the turbidimetric determinations (the paper electrophoretic results considered as reference) were tested according to the following formula: $Sy = \sigma_x \sqrt{1 - r^2}$ where σ_y equals the standard deviation as above and r is the correlation coefficient. For purposes of comparison the standard error of estimate is expressed in percentage of the mean.

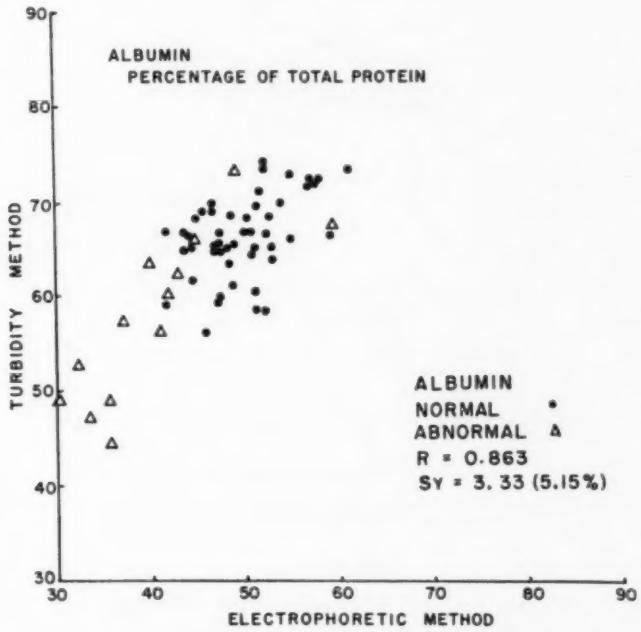


Fig. 1.—Comparison of albumin as determined by electrophoretic and turbidimetric methods.

RESULTS

1. Albumin (Fig. 1). The correlation is good, r being 0.863 and the standard error of estimate 5.15 per cent at the mean.

2. Albumin and alpha-1 globulin (Fig. 2). The correlation in general is good, r being 0.794 and the standard error of estimate 6.17 per cent at the mean.

3. Alpha-2 globulin (Fig. 3). The correlation is poor, r being 0.716 and the standard error of estimate 20.78 per cent at the mean.

4. Alpha globulin (Fig. 4). The correlation is poor, r being 0.734 and the standard error of estimate 20.21 per cent at the mean.

5. Beta globulin (Fig. 5). The correlation is poor, r being 0.588 and the standard error of estimate 16.88 per cent at the mean.

6. Gamma globulin (Fig. 6). The correlation is good, r being 0.864 and the standard error of estimate 16.32 per cent at the mean.

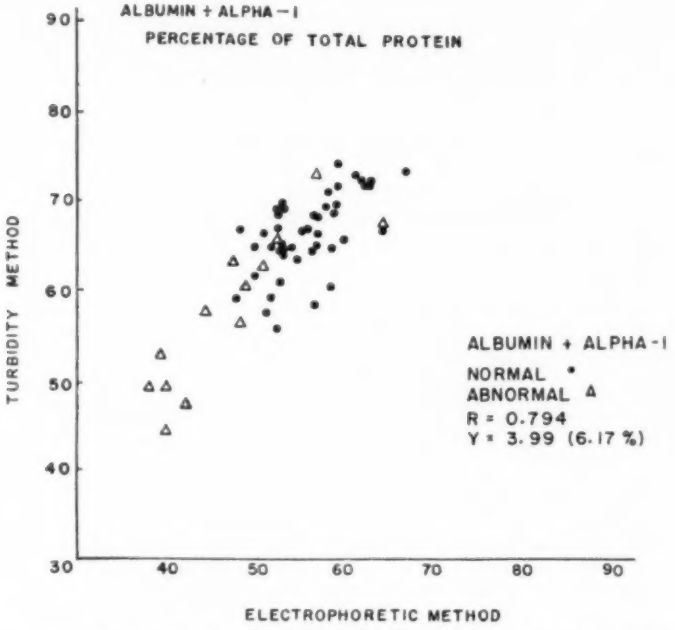


Fig. 2.—Comparison of albumin determined by turbidimetric method and albumin + alpha-1 determined by electrophoretic method.

DISCUSSION

Eriksen[21] compared free electrophoretic values of serum protein fractions with those of Wolfson-Cohn's sulfate-sulfite precipitation method.[9] He studied electrophoretic patterns on the various chemical fractions and found the albumin fraction to contain not only the nominal component but also a component indistinguishable from alpha-1 globulin, and traces of what appeared to be mainly parts of the alpha-2 globulin complex. He showed that in one case of beta-type multiple myeloma, a large part of the abnormal protein appeared in the gamma globulin chemical fraction although the electrophoretic pattern showed only a trace of gamma globulin.

Aull and McCord[17] suggested that their phosphate-turbidity method may correlate more closely with electrophoresis because they believe their method may measure certain nonprotein materials not measured by the Wolfson-Cohn method. Eriksen mentioned that since the chemical fractions are analyzed

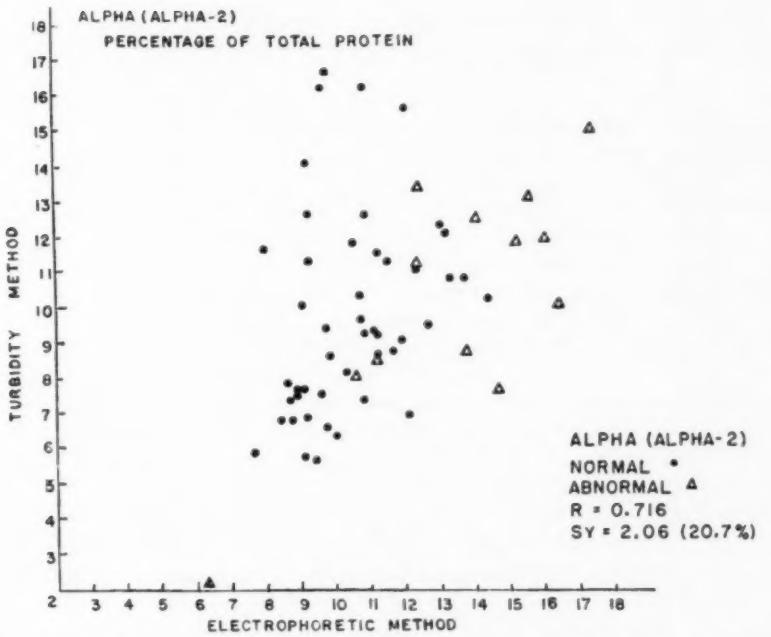


Fig. 3.—Comparison of alpha globulin as determined turbidimetrically with the alpha-2 globulin determined electrophoretically.

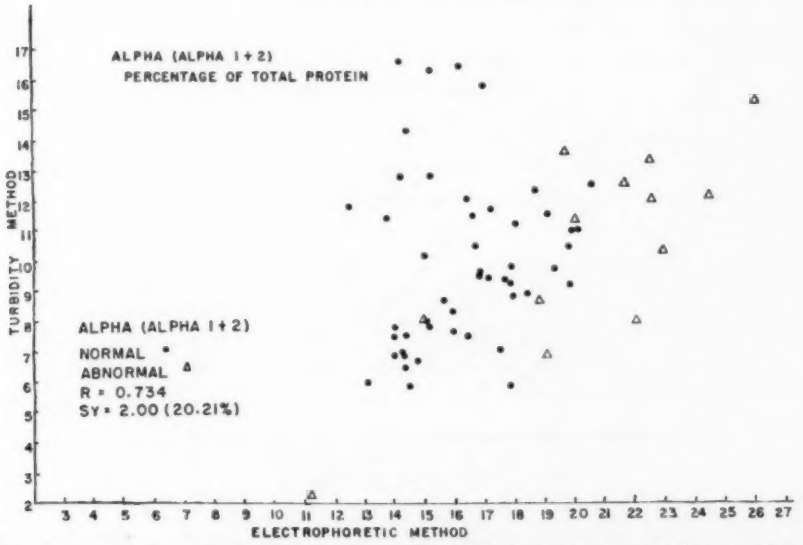


Fig. 4.—Comparison of alpha globulin determined by turbidity method and alpha-1 + alpha-2 determined by electrophoretic method.

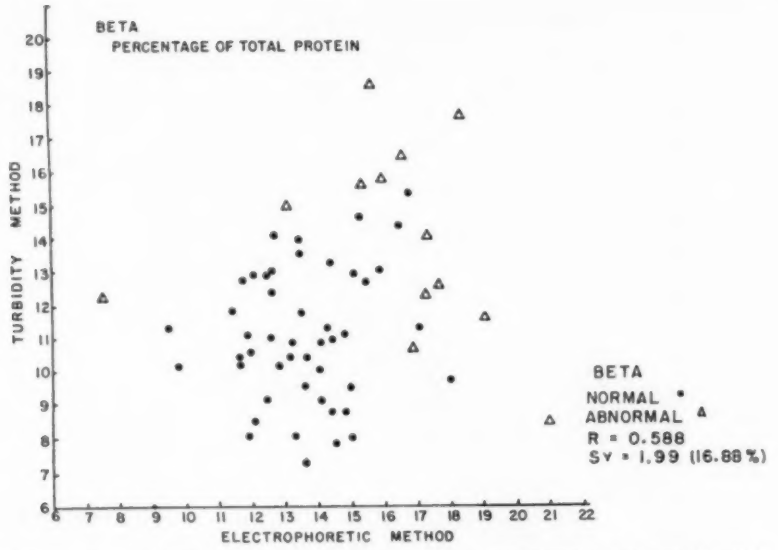


Fig. 5.—Comparison of beta globulin as determined by electrophoretic and turbidimetric methods.

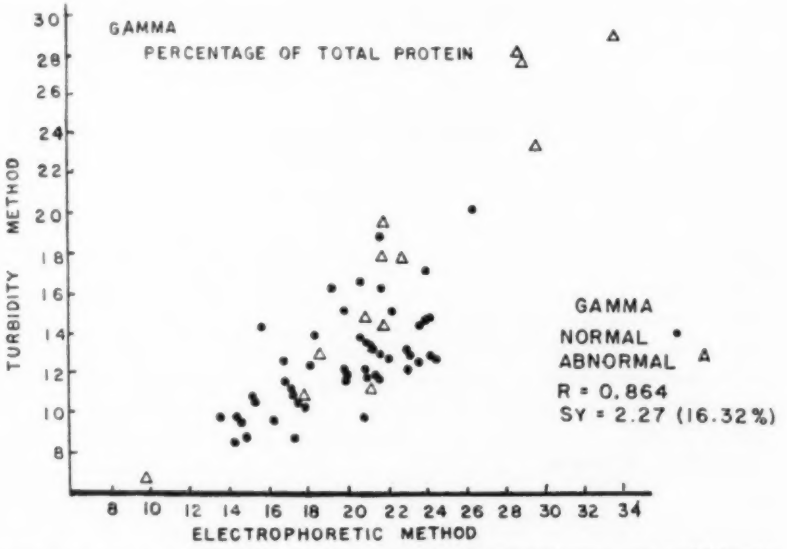


Fig. 6.—Comparison of gamma globulin as determined by electrophoretic and turbidimetric methods.

only for simple protein constituents one should then expect absolute agreement between chemical and electrophoretic methods only in the limiting case in which nonprotein material is absent.

By incorporating paper electrophoresis and bromphenol blue stain there is a direct relationship between dye uptake and serum proteins present which excludes lipoproteins, free lipid, and glycoproteins.[23] However, the beta globulin is still higher than that obtained turbidimetrically. This is a good indication that this increase in beta globulin is not mainly due to lipid moieties. Although lipoprotein accounts for 15 to 30 per cent of the beta globulin peak in normal sera and up to 60 per cent in abnormal sera when measured by refractive index increments,[16] the discrepancy that still remains between electrophoretic and chemical methods of determining serum protein fractionation is probably due to selectability of chemical fractionation, particularly with respect to the globulin fractions.

By combining the alpha-1 globulin determined electrophoretically with its albumin component the mean values between the electrophoretic and turbidimetric methods agree more favorably (mean difference changes from 16.6 to 9.5) even though the correlation is smaller, indicating that a portion of the alpha globulins are not precipitated with the globulin portion. The mean difference between the two methods is greater when the electrophoretically determined alpha-1 and alpha-2 globulins are combined and compared with the alpha globulins determined by the phosphate-turbidity method (mean difference changes from 1.3 to 7.4).

SUMMARY

The results of serum protein partition analysis evaluated by a statistical correlation between paper electrophoretic and phosphate-turbidimetric methods show poor correlation for the alpha and beta globulins although satisfactory results are obtained for albumin and gamma globulin.

Although it appears as though no direct correlation can be made between chemical and electrophoretic methods of serum protein partition analysis, Aull's phosphate-turbidity method is considered to be a valuable screening procedure for the detection of agammaglobulinemia or hypogammaglobulinemia.

REFERENCES

1. Durrum, E. L.: Abstracts of Papers, 115th meeting American Chemical Society, p. 22C; U. S. Army Medical Field Research Laboratory Report, Project 6-24-12-06(18), 1949; J. Am. Chem. Soc. **72**: 2943, 1950; J. Colloid Science **6**: 274, 1951.
2. Howe, P. E.: The Use of Sodium Sulfate as the Globulin Precipitant in the Determination of Proteins in Blood, J. Biol. Chem. **49**: 93-107, 1921.
3. Dole, V. P.: The Electrophoretic Patterns of Normal Plasma, J. Clin. Invest. **23**: 708-713, 1944.
4. Gutman, A. B.: The Plasma Proteins in Diseases, Advances Protein Chem. **4**: 155-250, 1948.
5. Marrack, J. R., and Hoch, H.: Serum Proteins; A Review, J. Clin. Path. **2**: 161-192, 1949.
6. Peterman, M. L., Young, N. F., and Hogness, N. R.: A Comparison of the Howe and the Electrophoretic Methods for the Determination of Plasma Albumin, J. Biol. Chem. **169**: 379-387, 1947.
7. Thorn, G. W., Armstrong, S. H., and Davenport, V. D.: Chemical, Clinical, Immunological Studies on the Products of Human Plasma Fractionation; The Use of Salt-Poor Concentrated Human Serum Albumin Solution in the Treatment of Hepatic Cirrhosis, J. Clin. Invest. **25**: 304-323, 1946.

8. Popper, H., de la Huerga, J. Franklin, M., Bean, W. V., Hall, W. D., Roth, J. I., and Schaffner, F.: Correlation Between Electrophoretic and Chemical Partitions of Serum Proteins, Am. J. Clin. Path. **20**: 530-538, 1950.
9. Wolfson, W. Q., Cohn, E., Calvary, E., and Ichiba, F.: Studies on Serum Proteins V Rapid Procedure for the Estimation of Total Protein, True Albumin, Total Globulin, Alpha Globulin, Beta Globulin, and Gamma Globulin in 1.0 ml. of Serum, Am. J. Clin. Path. **18**: 723-730, 1948.
10. Jager, B. V., and Nicherson, M.: Clinical Application of a Simple Method for Estimating "Gamma Globulins," J. Clin. Invest. **27**: 231-238, 1948.
11. Kibrick, A. C., and Blonstein, M.: Fractionation of Serum Into Albumin, and Alpha, Beta and Gamma Globulins by Sodium Sulfate, J. Biol. Chem. **176**: 983-987, 1948.
12. Majoor, C. L. H.: The Possibility of Detecting Individual Proteins in Blood Serum by Differentiation of Solubility Curves in Concentrated Sodium Sulfate Solutions, J. Biol. Chem. **169**: 583-594, 1947.
13. Martin, N. H., and Morris, R.: The Albumin/Globulin Ratio: A Technical Study, J. Clin. Path. **2**: 64-66, 1949.
14. Pillemer, L., and Hutchinson, M. C.: The Determination of the Albumin and Globulin Contents of Human Serum Methanol Precipitation, J. Biol. Chem. **158**: 299-301, 1945.
15. Reinhold, J. G., Bluemle, L. W., Jr., Campoy, F., Gilman, L., Gould, E. F., Martens, V. L., and Shuman, R.: Determination of Serum Proteins; Study of Agents for Separation of Albumin and Globulin, Fed. Soc. **8**: 242, 1949.
16. Lewis, L. A., and Page, I. H.: Electrophoretic and Ultracentrifugal Analysis of Serum Lipoproteins of Normal, Nephrotic, and Hypertensive Persons, Circulation **7**: 707-717, 1953.
17. Aull, J. C., and McCord, W. M.: A Simple Rapid Procedure for the Estimation of Albumin and Alpha, Beta, and Gamma Globulin in Serum, J. LAB. & CLIN. MED. **46**: 476-483, 1955.
18. Jencks, W. P., Durrum, E. L., and Jetton, M. R.: Paper Electrophoresis as a Quantitative Method: Serum Proteins, Biochem. J. **60**: 205-215, 1955.
19. Beckman Instruments Co., Inc. Operating Instructions, Spinco Model R., Spinco Division.
20. Gornall, A. G., Bardawill, C. J., and David, M. M.: Determination of Serum Proteins by Means of Biuret Reaction, J. Biol. Chem. **177**: 751-765, 1949.
21. Eriksen, N., Ellerbrook, L. D., and Lippincott, S. W.: Serum Protein Analysis by Electrophoresis and by the Wolfson-Cohn Chemical Method, Clin. Chem. **2**: 344-346, 1956.
22. Kenney, J. F.: Mathematics of Statistics, New York, 1939, D. Van Nostrand Company, Inc., chap. VIII.
23. Jencks, W. P., Smith, E. R. B., and Durrum, E. L.: The Clinical Significance of the Analysis of Serum Protein Distribution by Filter Paper Electrophoresis, Am. J. Med. **21**: 387-405, 1956.

FLUID FILM METHOD OF ELECTROPHORESIS

NEWTON RESSLER, PH.D., NORMAN A. NELSON, M.D., AND HAROLD D. OSTER, M.D.
ELOISE, MICH.

THE rapidly increasing interest in electrophoresis during the past few years has been largely attributed to free solution[1] or gelled[2] or paper[3] zonal methods. Another technique[4-6] combining some of the properties of both free solution and zonal procedures and suitable for routine use is described in this paper. This procedure utilizes, as the electrophoretic medium, a thin fluid film layered over a transparent plastic surface. The film consists of a trace of gelling agent dissolved in buffer. The quantity of gelling agent is sufficient only to impart a slight rigidity, not enough to cause gelation or loss of fluid characteristics. An investigation of some of the variables involved with this procedure, such as film thickness and rigidity, wick connections to electrode vessels, and humidity of the atmosphere above the film have been made. It is a simple matter to control these factors so that mobility is linear with time and the reproducibility comparable to other methods.[5] A relatively high degree of resolution can be achieved with the fluid film technique. It has the advantage that merely by drying the film after the electrophoresis, the proteins are fixed and available for staining on a background almost completely transparent.

It has been found that by using, as a surface upon which to layer the film, a single sheet of transparent plastic large enough for the simultaneous electrophoresis of many samples, the method is relatively simple and rapid, and appears convenient for routine clinical use. The application of this procedure to the electrophoresis of serum proteins is described below.

MATERIALS AND METHODS

A clear sheet of rigid vinyl,* 0.05 inch thick, has been found to provide a suitable surface upon which to layer the fluid film, being transparent, nonreactive, of uniform thickness, and with polished surfaces.

Cut the vinyl with scissors to the size of a supporting Pyrex or glass plate, 18 by 25 inches, and wash clean. Lay the vinyl sheet over the level Pyrex plate, which has been coated with mineral oil in order to prevent the vinyl from later warping under infrared heat. Clamp glass strips over the vinyl around the outside edges, to serve as borders to prevent the film from running off. Seal the joints with collodion. The later drying of the fluid is also facilitated by using cross strips to divide the plastic into 3 or 4 sections. Place electrode vessels along both lengths of the plastic and insert filter paper

From the Department of Medicine, Wayne County General Hospital, Eloise, Mich.
Received for publication Aug. 5, 1957.
*V.S.A., Bakelite Division of Union Carbide and Carbon, Ottawan, Ill.

wicks. The wicks consist of two layers of dampened Whatman No. 17 filter paper, wrapped together with Saran Wrap. Fill the electrode vessels with 0.03 M veronal buffer of pH 8.6 up to the level of the vinyl.

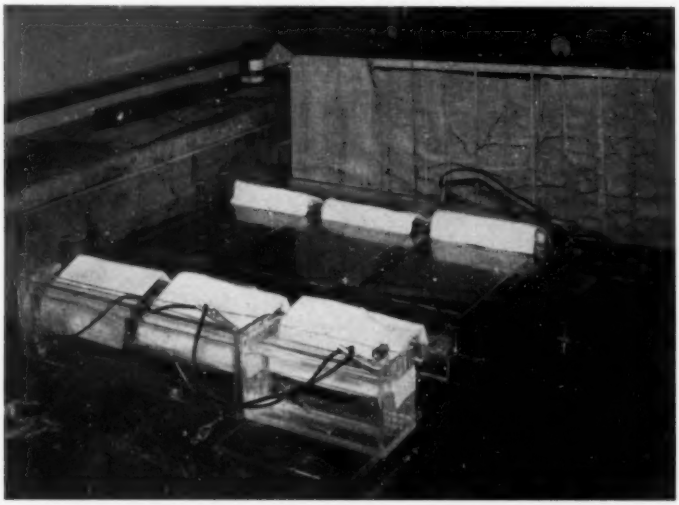


Fig. 1.—Fluid film apparatus ready to be covered and sealed. The electrode vessels and wicks can later be withdrawn so that the film can be dried without being moved.

The fluid film is prepared by adding to the veronal buffer, 0.13 Gm. per cent of Difco agar-agar, and dissolving by heating to a boil. Let cool to about 60° C., then add 200 ml. of this solution (0.17 ml. per cm.²) to the vinyl sheet. The fluid does not wet the vinyl and must be spread across the surface with a glass or plastic strip. Connect the wicks and apply a potential gradient of 5.7 volts per cm. while the solution is still warm. The serum can now be applied by means of a double thickness of Whatman No. 1 filter paper, 2 by 12 mm., soaked in the serum. By means of forceps, apply the wet paper to the film perpendicular to the electric field. Adequate room is available for 12 serum samples. Fig. 1 shows 12 samples laid on a fluid film. The film is retained on the vinyl by glass strips clamped around the edges. The filter paper wicks connect to electrode vessels containing buffer at the same level as the film. A cover consisting of wet blotting paper held to a glass plate by means of rubber bands is positioned over the apparatus, and the remaining openings are closed with Saran Wrap. After running for a period of 4½ hours, remove the cover and the filter paper wicks and store for reuse. Without disturbing the film, remove the electrode vessels and turn on six 250 watt infrared bulbs positioned 18 inches above the film, so as to cover the area equally. In order to prevent overheating, cover the dry areas which develop as the heating proceeds with cardboard.

The fluid film dries into a transparent protective coating over the proteins. When dry, gently immerse the vinyl sheet in the dye for 30 minutes. The dye is an alcoholic solution containing 1 Gm. bromphenol blue and 100 Gm. mercuric chloride per liter. Immerse the film in a 1 per cent aqueous solution of acetic acid for 3 minutes and dry at room temperature. Finally immerse in an aqueous solution containing 10 per cent acetic acid and 1 per cent sodium acetate for 10 minutes. Let dry again at room temperature, then cut the vinyl with scissors into individual electrophoretograms. Any slight turbidity in the background can be eliminated by spraying with a clear acrylic plastic.*

*Plasti-Kote, Inc., Cleveland, Ohio.

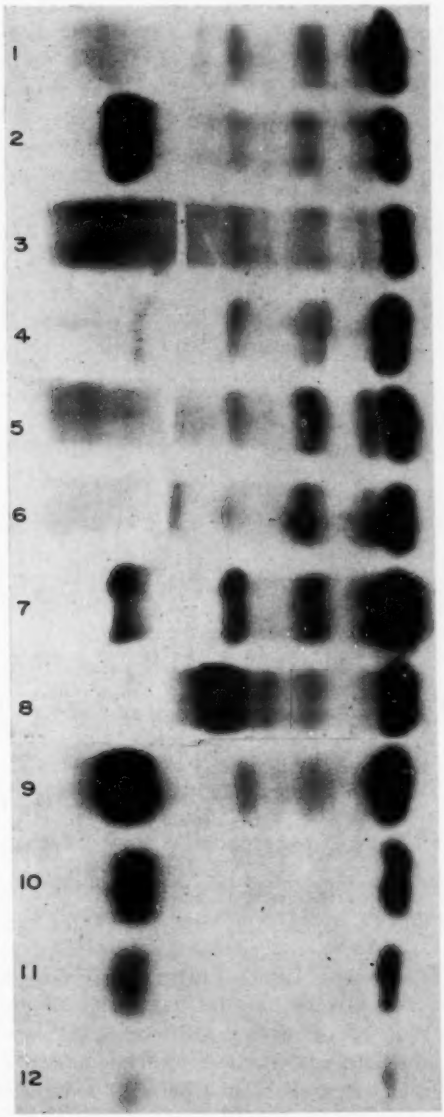


Fig. 2.—Electrophoretograms of 9 different pathologic sera obtained simultaneously on a single vinyl sheet. The diagnoses corresponding to the individual sera are: (1) neuroblastoma, (2) multiple myeloma, (3) Laennec's cirrhosis, (4) subacute lymphocytic leukemia with hypogammaglobulinemia, (5) metastatic carcinoma, (6) agranulocytosis, (7) multiple myeloma, (8) multiple myeloma; 9 through 12 were of the same serum (multiple myeloma) but diluted with buffer before application, 1:1, 1:2, 1:4, and 1:8, respectively.

RESULTS

Twelve electrophoretograms, representative of the various pathologic states indicated, obtained on a single vinyl sheet by the above procedure are shown in Fig. 2. Nos. 9 through 12 represent the same serum, but they are diluted progressively with buffer before application to show the effect of decreasing protein concentration. Fig. 3 shows the corresponding densitometric curves of samples 1 through 8, obtained by means of a Kelab densitometer.

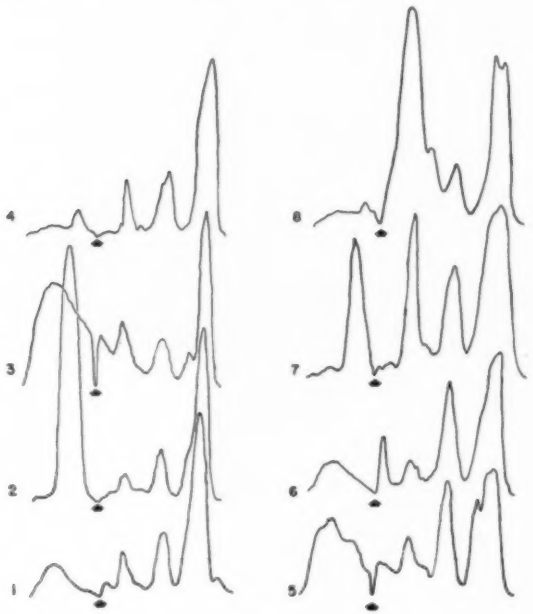


Fig. 3.

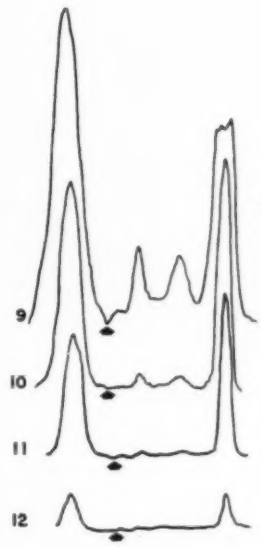


Fig. 4.

Fig. 3.—Densitometric curves corresponding to electrophoretograms 1 through 8, of Fig. 2; points of serum application are indicated by arrows.

Fig. 4.—Densitometric curves corresponding to the electrophoretograms 9 through 12, Fig. 2.

The curves of 9 through 12 (Fig. 4) illustrate a decrease in the width of the bands of the more concentrated proteins, especially albumin, with decreasing concentration. In order to compare the difference between an electrophoretogram rinsed only in a 1 per cent solution of acetic acid to one also rinsed in a solution of 10 per cent acetic acid and 1 per cent sodium acetate, a sample of serum was subjected to electrophoresis for a period of 16 hours. The longer period of time was used in order to obtain a greater degree of resolution. In Fig. 5, the bottom electrophoretogram and densitometer curve show the results obtained after the first rinse only. The upper electrophoretogram and densitometer curve corresponds to the same sample after rinsing in the second solution.

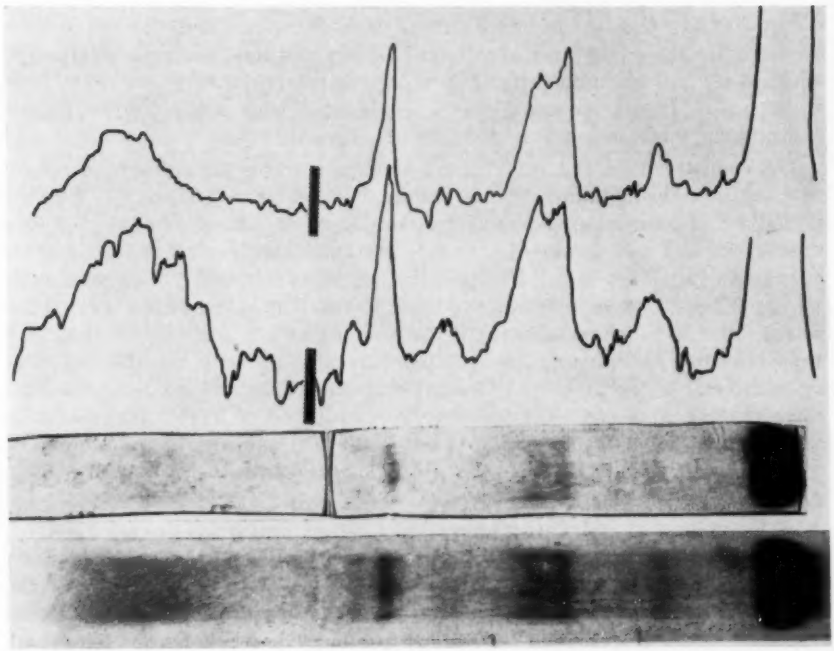


Fig. 5.—Electrophoretograms and densitometric curves of a sample of serum subjected to electrophoresis for a period of 16 hours at a potential gradient of 3.3 volts per centimeter. The bottom electrophoretogram and curve were obtained before rinsing with a solution of 10 per cent acetic acid and 1 per cent sodium acetate. The top electrophoretogram and curve were obtained after this rinse. (The electrophoretogram was cut into 2 sections in order for it to fit the densitometer frame.)

DISCUSSION

The fluid film technique requires no special apparatus, and takes little time per sample when a large number are done simultaneously on a single plastic sheet. A glass sheet could be substituted for the plastic, but would be less convenient for handling and cutting. The operations are simple to perform, but certain critical factors should be controlled for best results. These include: (1) leveling the plane of the plastic, (2) having the buffer in the electrode vessels at the same height as the film, (3) using the film promptly after preparation, (4) keeping the film sealed during electrophoresis, and (5) drying evenly afterward. If the level of the buffer is not the same as the film, drainage effects will occur through the wicks. If the fluid film is allowed to stand after being spread, but before applying the current, it will tend to become more rigid, and the results will be less successful. If the film is not covered during electrophoresis, the buffer composition will be altered by evaporation. By observing these precautions, a technician can easily do 12 to 16 samples per day with consistent and reproducible results.

The 0.03 M veronal buffer is less concentrated than is generally used for routine purposes.[7] The resolution obtained by the present method is not

diminished by the use of more dilute veronal buffer, and the lower ionic strength has the advantages of greater mobility and less heat generation. The albumin alpha-1 globulin separation is less complete, however, at lower ionic strength. A greater separation can be obtained when desired by either increasing the ionic strength or the time of migration.

A comparison of Fig. 5 to Figs. 2 and 3 illustrates the increase in resolution that can be obtained by increasing the time of migration. A running period of as long as 16 hours is technically more difficult, however, and a time of from 4 to 6 hours can provide resolution sufficient for most routine purposes. Fig. 5 also illustrates that the last rinse solution, 10 per cent acetic acid and 1 per cent sodium acetate, tends to result in a loss of the minor components but is of more uniform shade and presents a greater contrast (not apparent from the photograph). It appears preferable to use the last rinse when interest is restricted to principal components, but should be omitted for more general purposes. The protein concentration also affects the resolution as shown in Fig. 4. It appears that only a limited quantity of protein can be oriented by the fluid film since the widths of the bands increase with protein concentrations above a certain level.

The ability of the fluid film to serve as an anticonvection medium for electrophoresis appears related to its retention of the characteristics of a fluid, i.e., to assume the shape of the vessel it occupies.[8] When the rigidity of the film is increased by increasing the agar concentration, by conducting the electrophoresis in a cold room, or by letting the film stand and harden before applying the current, the resolution and mobility then decrease.[5] Consequently, it appears possible that the fluid film may act by virtue of relaxation phenomena, i.e., resisting thermal motions of short duration, while submitting to constant electric forces.[9]

SUMMARY

A procedure has been described for a method of electrophoresis designed for routine purposes and employing as an electrophoretic medium a thin fluid film. By layering the latter over a transparent vinyl sheet of suitable size, any convenient number of samples can be done simultaneously. By simply drying the film on the plastic after electrophoresis, the proteins are fixed for staining on a clear plastic background. The transparency of the plastic is an advantage for purposes of densitometric recording. By observing some simple precautions, consistent results can easily be obtained. Factors affecting the resolution of the method are discussed.

We are indebted to Dr. Samuel D. Jacobson, Physician-in-Chief of Wayne County General Hospital, for his stimulating interest and support. We also gratefully acknowledge the technical assistance of Miss Virginia Bodick and Mr. Thomas Moy.

REFERENCES

1. Tiselius, A.: A New Apparatus for Electrophoretic Analyses of Colloidal Mixtures, Tr. Faraday Soc. 33: 524, 1937.
2. Gordon, A. H., Keil, B., Sebesta, K., Knessl, O., and Sorm, F.: Coll. Czechoslov. chem. commun. 15: 1, 1950.

3. Block, R. J., Durrum, E. L., and Zweig, G.: A Manual of Paper Chromatography and Paper Electrophoresis, New York, 1955, Academic Press, Inc.
4. Ressler, N., and Jacobson, S. D.: Electrophoresis of Serum Proteins in a Viscous Film, Science **122**: 1088, 1955.
5. Ressler, N., and Zak, B.: Electrophoresis in a Fluid Film, Clin. chim. Acta. **1**: 392, 1956.
6. Ressler, N., and Jacobson, S. D.: Effect of Buffer on Fluid Film Electrophoresis, Clin. chim. Acta. **2**: 372, 1957.
7. Laurell, C. B., Laurell, S., and Skoog, N.: Buffer Composition in Paper Electrophoresis, Clin. Chem. **2**: 99, 1956.
8. Glasstone, S.: Elements of Physical Chemistry, New York, 1948, D. Van Nostrand Company, p. 147.
9. Kruyt, H. R.: Colloid Science, Amsterdam, 1945, Elsevier Press, Inc., vol. 2.

A SIMPLE NEPHELOMETRIC TEST FOR BETA LIPOPROTEINS OF HUMAN SERUM

H. N. Antoniades, Ph.D., J. L. Tullis, M.D., L. H. Sargeant, M.D.,
R. B. Pennell, Ph.D., and J. L. Oncley, Ph.D.
Boston, Mass.

THE use of dextran sulfate for the isolation of β-lipoproteins directly from human plasma, or from Cohn's fractions II + III and III – O, was introduced by Oncley, Walton, and Cornwell.[1] The method is based on an interaction between the dextran sulfate and the β-lipoproteins which forms a precipitate consisting of a dextran sulfate β-lipoprotein complex. Removal of fibrinogen from the plasma is necessary prior to this precipitation, since fibrinogen also precipitates with dextran sulfate. Based on this original observation, an attempt was made in the present study to develop a simple nephelometric test to measure the quantitative variation of β-lipoproteins in human blood serum. In order to standardize the nephelometric readings, 2 preparations of pure human β-lipoproteins (Sf 3-9 and 10-100) were studied.

METHODS AND MATERIALS

The interaction of dextran sulfate and β-lipoprotein to form an insoluble complex is effected by addition of dextran sulfate to serum by the formation of a precipitate consisting of a dextran sulfate β-lipoprotein complex. This interaction is effected by a variety of factors: pH, temperature, ionic strength, β-lipoprotein concentration, and dextran sulfate concentration. When small amounts of dextran sulfate and β-lipoproteins interact, turbidity occurs. At constant concentrations of dextran sulfate, the turbidity depends on the amount of the β-lipoproteins present. Measurement of this turbidity through a nephelometer can then express the changes of the β-lipoprotein content of the serum. Since the development of this turbidity depends on many factors, a careful examination of the conditions of the interaction was undertaken.

Dextran Sulfate.—The dextran sulfate employed for this purpose was prepared from dextran which had an intrinsic viscosity (n) of 0.65 by the method of Ricketts.[2] The sulfur content was between 16.5 and 17.4 per cent. Dilutions were made with 0.15 M sodium chloride to which 5 per cent by volume of 0.15 M sodium citrate was added.

Beta Lipoprotein Preparations.—The following β-lipoproteins[1] prepared from fresh plasma by ultracentrifugation were used: Sf 3-9. This lipoprotein, consisting of 55 to 65 per cent of the total β-lipoproteins of human plasma, contains: peptide, 21.9 per cent; phospholipids, 22.4 per cent; total cholesterol, 30.8 per cent; cholesterol phospholipids ratio, 1.38[1]; Sf 10-100, consisting of 25 to 35 per cent of the β-lipoproteins, contains peptide, 10.0 per cent; phospholipids, 19.0 per cent; total cholesterol, 16.0 per cent; neutral fat, 49 per cent; cholesterol phospholipids ratio, 0.85.[1]

From the Protein Foundation, Inc., Jamaica Plain, Mass., New England Deaconess Hospital, and the Departments of Medicine and Biological Chemistry, Harvard Medical School, Boston, Mass.

Supported in part by grants from the U. S. Public Health Service, from the Kresge Foundation, other charitable trusts, and by contributions from companies engaged in business interested in the alleviation or cure of human diseases.

Received for publication Aug. 14, 1957.

Nephelometric Determinations.—These were performed with a Mueller nephelometer. This instrument can be briefly described as follows: the light source is a mercury vapor lamp (General Electric H4 lamp). The light is passed through a Corning No. 3486 filter (polished) of approximately 1/16 in. thickness and focused on the sample with a lens of about 10 cm. focal length. The sample is placed in a clear glass bottle, outer diameter about 2.9 cm., which is immersed in a water bath to decrease the amount of light scattered at the surface. The sample holder is a blackened brass cylinder suitably milled with slots at 0°, 45°, and 180° from the emergent light beam fitted with blackened light baffles. The water bath is fitted with plain glass windows at 0°, 45°, and 180° from the emergent light beam. A neutral filter of suitable density (a piece of partially exposed photographic film) is placed in the 0° window to make the transmitted light comparable in intensity to the 45° transmission. The light at 45° is restricted to that emerging from a slit approximately ⅜ in. wide and ⅞ in. long. Measurement is made of the light that has passed through a sample at 0° and at 45°, using a photocell and amplifier (Photovolt Meter, Model 501A) which can be placed in these two positions. The ratio (light at 45°/light at 0°), of the unknown is compared to the ratio (light at 0°/light at 45°) of a silica standard* having an assigned value of 27 nephelometric units. The results are converted to nephelometric units according to formula:

$$\frac{0^\circ \text{ Rdg.}}{45^\circ \text{ Rdg.}}\ \text{standard} \times 27 \times \frac{45^\circ \text{ Rdg.}}{0^\circ \text{ Rdg.}}\ \text{unknown} = \text{nephelometric units.}$$

EXPERIMENTAL

Effect of Dextran Sulfate and β-Lipoprotein Concentration.—One part (0.3 ml.) of various dilutions of β-lipoprotein solution (Sf 3-9) was added to 50 parts (15 ml.) of a dextran sulfate solution at pH 7.1 and ionic strength 0.15. After mixing well, the solution was left at room temperature for 25 to 30 minutes and then the developed turbidity was measured at 0° and 45°. Fig. 1 presents the results obtained as a function of dextran sulfate concentration and β-lipoprotein concentration, the other factors remaining constant. It is clear that both β-lipoprotein and dextran sulfate concentration effect the final results. Concentrated dextran sulfate solutions produced lower turbidities and very dilute solutions interacting with concentrated β-lipoprotein solutions produced anomalous readings. Similar effect was observed with pure β-lipoprotein preparation of Sf 10-100 (Fig. 2).

Effect of Temperature and Time.—Differences in the temperature can also alter the turbidity, all other factors remaining constant. The turbidity at room temperature is constant 25 to 50 minutes after the addition of the β-lipoproteins to the dextran sulfate solution, and then slowly decreases (Fig. 3). A pH of 7.1 ± 0.1 and an ionic strength of 0.15 have been used in these experiments.

Effect of Gamma Globulins and Albumin.—Gamma globulin and albumin of high purity, prepared from human plasma, were added, respectively, to dextran sulfate solution under the same conditions described for the β-lipoprotein interaction in concentrations from 0.5 to 2.5 Gm. per cent. The turbidity obtained was negligible indicating that there is no interference of γ-globulin and albumin in the β-lipoprotein dextran sulfate interaction.

Conditions and Method for the Nephelometric Determination of the β-Lipoproteins Levels of Human Blood Serum.—The conditions and the method for the nephelometric determination of the β-lipoproteins of human serum† are as follows.

*The nephelometer standard has been made by diluting a solution of colloidal silica so that the optical density of a 10 cm. length of the solution is 0.118 at 5461 Å, as measured in the Beckman spectrophotometer.[3] This material has been shown to scatter light in accordance with Rayleigh's law for small particles, and this optical density corresponds to an absolute turbidity of 0.0271 cm.$^{-1}$. This solution has been assigned a value of 27 nephelometric units in the Mueller nephelometer. Thus, one nephelometric unit in the Mueller nephelometer corresponds to 0.0010 cm.$^{-1}$ absolute turbidity.

†Blood serum should preferably be collected in the morning before any food is taken and must be free of hemolysis.

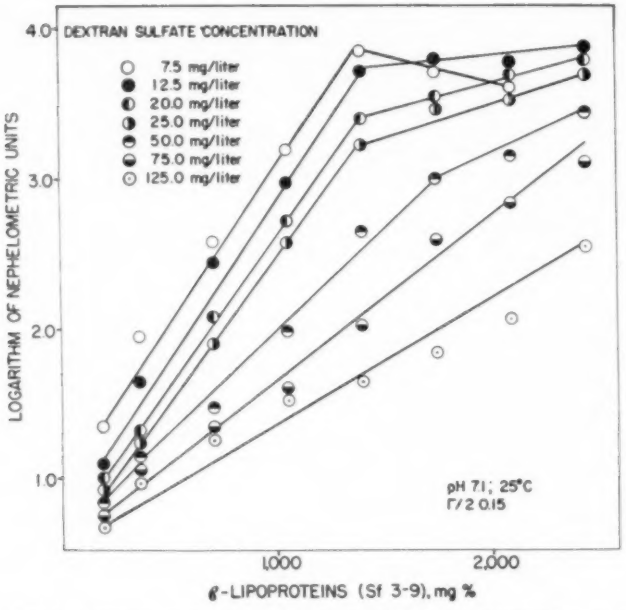


Fig. 1.—Effect of dextran sulfate and β-lipoprotein (Sf 3-9) concentration on the development of turbidity.

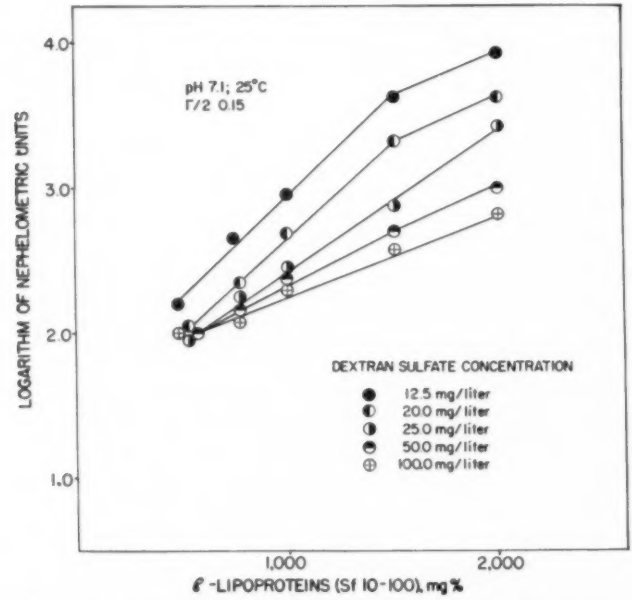


Fig. 2.—Effect of dextran sulfate and β-lipoprotein (Sf 10-100) concentration on the development of turbidity.

Stock Dextran sulfate solution: Five-tenths gram of dextran sulfate prepared according to Ricketts' method as described above are dissolved in 100 ml. of distilled water. The solution, divided in small portions, is kept frozen to preserve it from bacterial growth.

Dextran sulfate reagent: Eight-tenths milliliter of the stock solution is added to 200 ml. of 0.15 M sodium chloride solution which contains 5 per cent by volume 0.15 M sodium citrate solution. The solution is mixed well before use and is kept at 2° C. The pH is 7.1 ± 0.1.

Method: Fifteen milliliters of the dextran sulfate reagent is placed at room temperature in a clear glass bottle. Three-tenths milliliter of serum is added, the solution is mixed well and is left for 25 to 30 minutes at room temperature for the development of the turbidity.

Measurement of turbidity: The turbidity is measured in Mueller's nephelometer by comparison of the light transmitted at 0° and the light scattered at 45°. The result is expressed in nephelometric units as described above.

Experimental results indicate that lipemic sera do not affect the nephelometric readings of Mueller's nephelometer.

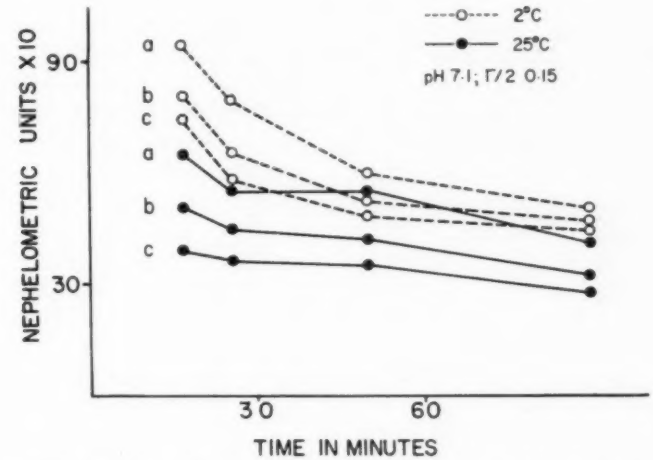


Fig. 3.—Effect of time and temperature on turbidity; *a*, *b*, and *c* represent samples of human serum.

RESULTS

In order to establish normal limits of β-lipoprotein levels with the present technique, the serums of 104 normal volunteer male and female subjects, 18- to 80-years-old, were used. The blood serum was collected from either fasting subjects or those who had a light breakfast. The results obtained are shown in Table I. The normal subjects showed a range between 50 and 700 nephelometric units (0.05 to 0.7 cm^{-1} absolute turbidity); however, 84 per cent of the normal subjects showed 100 to 400 units. Fourteen per cent showed 500 to 700 units. Table I indicates the relation between the obtained nephelometric units and the equivalent β-lipoprotein concentration according to Fig. 1. This relation is only approximate and is referred to β-lipoproteins of Sf 3-9 since

a variety of β-lipoproteins exist in human serum and their interaction with a given solution of dextran sulfate may vary with the individual β-lipoprotein (see Figs. 1 and 2).

TABLE I. NEPHELOMETRIC UNITS OBTAINED FROM THE EXAMINATION OF THE SERUM OF 104 NORMAL INDIVIDUALS AND THEIR RELATION TO THE β-LIPOPROTEIN CONCENTRATION ACCORDING TO FIG. 1

NEPHELOMETRIC UNITS	CASES (PER CENT)	β-LIPOPROTEINS* (SF 3-9) MG. PER CENT
50 to 100	2	300 to 390
100 to 200	25	390 to 480
200 to 300	29	480 to 510
300 to 400	30	510 to 600
400 to 500	6	600 to 650
500 to 600	5	650 to 680
600 to 700	3	680 to 780

*Dry weight.

Clinical Value of the Present Nephelometric Test.—The changes of the β-lipoprotein levels in human serum can be determined through the nephelometric technique as described above. Since this is a simple and rapid test, the blood serum of a large number of individuals can be examined with this method in order to study whether a specific relation exists between the β-lipoprotein of the serum and the clinical observations. The results obtained are shown in Table II.

TABLE II. NEPHELOMETRIC UNITS IN VARIOUS DISEASES

CASES*	NUMBER OF CASES	NEPHELOMETRIC UNITS
Diabetes under control	40	110 to 675
Diabetes with arteriosclerosis and hypertension	20	800 to 7,750
Uncontrolled diabetes	1	1,650
Two weeks after insulin treatment		490
Pregnant diabetics with vascular complications	8	800 to 3,200
Nondiabetic arteriosclerosis	3	1,125 to 3,550
Alcoholic cirrhosis	3	1,000 to 3,000
Cancer of liver and Laennec's cirrhosis	7	250 to 675
Various diseases without vascular complications	30	150 to 700

*Many of the sera were made available by the Joslin Clinic for which we wish to express grateful acknowledgment.

Diabetes.—Sixty cases of diabetes were examined. Well-controlled diabetic patients without clinically observed disturbances were found to have nephelometric units within the normal range (110 to 675 units) (Table II). In the few cases of nondiabetic arteriosclerosis examined, the nephelometric units were similarly increased from 960 to 2,350 units. A similar result was obtained with 8 pregnant diabetic women with vascular complications. In one new, uncontrolled diabetic subject the nephelometric units were increased (1,650 units). Two weeks later when the patient was under insulin control the units were normal (490 units).

In 10 diabetic patients the β-lipoproteins of the serum were determined with paper electrophoresis, and the relative results of the electrophoretic examinations and the nephelometric units are shown in Table III. It is clear that

when the β-lipoprotein content in the serum was electrophoretically increased, the nephelometric units were also high. It is interesting to note that when the β-lipoproteins of sample 10 were electrophoretically normal but the γ-globulins very high, the nephelometric units were within the normal limits, reconfirming the experiment indicating that the γ-globulins do not effect the interaction.

TABLE III. COMPARISON OF THE NEPHELOMETRIC UNITS OF SERUM SAMPLES WITH THEIR β-LIPOPROTEIN CONTENT, CALCULATED FROM PAPER ELECTROPHORESIS

SAMPLE	NEPHELOMETRIC UNITS (NORMAL: 500-700)	β-LIPOPROTEIN LEVELS (NORMAL: 4-6)*	γ-GLOBULIN LEVELS (NORMAL: 14-15)
1	100	5.0	-
2	250	7.1	-
3	325	6.0	-
4	350	8.0	-
5	600	8.7	-
6	1,200	12.4	-
7	3,050	20.2	-
8	3,550	17.1	-
9	3,800	22.0	-
10	250	5.8	26

*Values kindly made available by Dr. Pierre Moinat of the New England Deaconess Hospital.

Liver Diseases.—Increase in nephelometric units has been observed in 3 cases of alcoholic cirrhosis of the liver and 2 cases of malignant melanoma with extensive liver involvement (1,125 to 3,550 units). In 4 cases of cancer of the liver and 3 of Laennec's cirrhosis the nephelometric units were within the normal units.

In 32 cases of various diseases without vascular complications or increased cholesterol, the nephelometric units were normal. The above preliminary observations suggest that a close relation exists between the increase of the nephelometric units and vascular complications of diabetic or nondiabetic subjects, and probably with certain liver complications. Since this method is specific and simple an extended clinical study may be of diagnostic value.

DISCUSSION

The β-lipoproteins of human plasma have been the subject of extended studies, from the physical chemical point of view as well as the clinical. They are unstable protein complexes carrying cholesterol in the blood stream and their preparation and characterization from fresh plasma is carried out under well-controlled conditions.[4] The observation that changes of β-lipoprotein levels exist in certain clinical cases of diabetes and arterial diseases led to an attempt for the development of techniques for the determination of the β-lipoproteins in human plasma or serum. Fractionation microtechniques have been developed for the preparation of the β-lipoproteins from small amounts of human plasma.[5] However, these methods are complicated and only a limited number of samples can be determined at the same time. Determination of the β-lipoprotein index through the ultracentrifuge techniques are successfully applied[6]; but, application for a routine test requires highly trained personnel and expensive equipment.

The present technique, as described above, is based on a specific β-lipoprotein dextran sulfate interaction which, under controlled conditions, is used for an accurate determination of the β-lipoprotein levels. The albumin and the γ-globulins of human serum do not interfere in this interaction. The specificity of the test has been established with the use of pure β-lipoprotein preparations, and comparative studies with results obtained electrophoretically. The rapidity and simplicity of the test is a great advantage for a routine clinical application of the method. It is worth-while to note that only a small amount of serum (0.3 ml.) is required for the test.

The β-lipoproteins of Sf 3-9, which represent 55 to 65 per cent of the total, were found to be more sensitive to this method than the β-lipoproteins of Sf 10-100 (Figs. 1 and 2) which represent 25 to 35 per cent. A change of the ratio of these lipoproteins may effect the result on the nephelometric units. An increase of the Sf 3-9 will produce higher nephelometric units whereas an equal increase of the Sf 10-100 lipoproteins will increase the nephelometric units to a lesser extent. However, the differences in sensitivity are not so great as to alter the results significantly in cases where the ratio of these two lipoproteins may change.

Increase of the total serum cholesterol does not necessarily mean an increase of the β-lipoproteins; instead, an increase of the alpha lipoproteins is possible. On the other hand, in cases with normal total cholesterol an increase of the β-lipoproteins may exist, especially when the alpha lipoproteins are low and most of the cholesterol binds with the β-globulins. In 2 cases out of 20 with high cholesterol levels the β-lipoproteins were found normal; in 3 cases with normal total serum cholesterol the β-lipoproteins were increased.

In the present study the dextran sulfate which was used was prepared by Ricketts' method.[2] Interaction between β-lipoproteins and various other sulfated polysaccharides of different sulfur content and molecular weight is also possible.[7] Therefore, when other preparations are used for the interaction with the β-lipoproteins, different from the one used in this study, it is obvious that new nephelometric units for normal values must be established.

SUMMARY

A simple and rapid technique for the nephelometric determination of the β-lipoproteins of human blood serum has been described. The method is based on a specific interaction between the β-lipoproteins of serum and dilute solutions of dextran sulfate which results in the development of a turbidity. The turbidity depends on the amount of β-lipoproteins of serum, all other factors remaining constant. The turbidity is measured with Mueller's nephelometer and is expressed in nephelometric units. Control experiments with two pure β-lipoprotein preparations from human plasma of Sf 3-9 and 10-100, and comparative measurements with electrophoretic data on the amount of the β-lipoprotein examined in human blood serum established the specificity and accuracy of the described technique.

The normal range of nephelometric readings was studied by examining the sera of 104 normal subjects. A preliminary attempt for a clinical evaluation of this method indicated a close relation between the increase of the nephelometric units and arteriosclerosis, vascular complications of diabetes and certain cases of liver involvement.

REFERENCES

1. Oncley, J. L., Walton, K. W., and Cornwell, D. G.: A Rapid Method for the Bulk Isolation of β-Lipoproteins From Human Plasma, J. Am. Chem. Soc. **79**: 4666-4671, 1957.
2. Ricketts, C. R.: Dextran sulphate—A synthetic analogue of heparin, Biochem. J. **51**: 129-133, 1952.
3. Edelhock, H., Katchalski, E., Maybury, R. H., Hughes, W. L., Jr., and Edsall, J. T.: Dimerization of Serum Mercaptalbumin in Presence of Mercurials. I. Kinetic and Equilibrium Studies With Mercuric Salts, J. Am. Chem. Soc. **75**: 5058-5072, 1953.
4. Oncley, J. L., Melin, M., Richert, D. A., Cameron, J. W., and Gross, P. M., Jr.: Preparation and Properties of Serum and Plasma Proteins. XIX. The Separation of the Antibodies, Isoagglutinins, Prothrombin, Plasminogen and β-Lipoprotein Into Subfractions of Human Plasma, J. Am. Chem. Soc. **71**: 541-550, 1949.
5. Lever, W. F., Gurd, F. R. N., Uroma, E., Brown, R. K., Barnes, B. A., Schmid, K., and Schultz, E. L.: Chemical, Clinical and Immunological Studies on the Products of Human Plasma Fraction. XL. Quantitative Separation and Determination of the Protein Components in Small Amounts of Normal Human Plasma, J. Clin. Invest. **30**: 99-111, 1951.
6. Gofman, J. W., Jones, H. B., Lindgrey, F. T., Lyon, T. P., Elliott, H. A., and Strisower, B.: Blood Lipids and Human Atherosclerosis, Circulation **2**: 161-178, 1950.
7. Bernfeld, P.: Properties of Purified Human Serum β-Lipoglobulin, Fed. Proc. **16**: 153, 1957.

A MODIFIED METHOD FOR EVALUATION OF CLINICAL USEFULNESS OF ANTIBIOTICS

ILSE HOETTE, M.S., AND ADRIANUS P. STRUYK, PH.D.
DELFT, HOLLAND

DETERMINATION of the sensitivity of microorganisms associated with infections against the action of divergent antibiotics is currently an accepted routine procedure in most clinical laboratories and hospitals. In fact, such a test is a "must" in order to treat a patient intelligently with the right type of antibiotic. In general there are two methods by which this can be achieved: the so-called "dilution method" and the "agar diffusion method."

For routine work the latter method is much preferred because of its ease of application, and its speedy results. No pure culture is required and usually smears with infected material, taken directly from the patient, may be used. The required information regarding the correct antibiotic for treatment is usually obtained in less than 24 hours.[1-7] Most frequently this test is performed with dried standardized paper disks, each one impregnated with a definite amount of an antibiotic. The comparative zone sizes obtained are usually taken as a measure for the sensitivity of the infective organism for the different antibiotics.

It cannot be denied that these prepared disks have given quite satisfactory and practical results. However, there are a number of disadvantages to this method which should not be underestimated since there are many factors which influence the zone size. Misinterpretation of these factors might easily lead to faulty conclusions. For instance two strains may have identical sensitivity for a certain antibiotic (as determined by dilution test), yet the zone size, determined under comparable conditions, may vary widely. Factors such as composition of the nutrient agar, thickness of the agar layer, amount of inoculum used, speed of growth of the test organism, differences in retention by the type of paper used, speed of diffusion of the antibiotic into the medium, and the keeping quality of the antibiotic in dry form as well as during diffusion all play a role of paramount importance on the ultimate size of the zone of inhibition.

An attempt was made to standardize the procedure of the paper disk method by eliminating as many variables as feasible. The ultimate purpose was to design a method by which the clinician may obtain the correct information about what would be the antibiotic of choice. In addition, however, he must be assured that the usual dosage of this antibiotic gives a blood level high enough to combat the disease successfully.

From the Antibiotic Division, Research Laboratories of the K.N.G & S. F. Royal Netherlands Fermentation Industries, Ltd., at Delft, Holland.
Received for publication July 25, 1957.

The method ultimately arrived at is based on the size of the zone of inhibition observed under highly standardized conditions. In case this zone size is larger than 10 mm. the antibiotic under consideration is expected to give clinically reliable results. However, in case the zone size is smaller than 10 mm. the usual therapeutic dosage of this antibiotic administered to a patient is expected to be ineffective for proper treatment and the use of such an antibiotic is contraindicated.

MATERIALS AND METHODS

Standardization of Technique.—It was obvious that standardization had to include such factors as type and size of paper disk, amount of antibiotic per disk, type of nutrient agar, and thickness of layer as well as other pertinent factors.

Filter Paper Disks.—Disks with a 6 mm. diameter were selected; they were punched from sheets of Whatman No. 3 filter paper, sterilized for 1½ hours at 125° C., and stored in a dry place. When needed, a number of these disks are spread evenly over a sterile microscope slide using sterile forceps.

Fig. 1.—Micropipette.

Micropipette for Distribution of Antibiotic Solutions on Disks.—Solutions of known concentration of the antibiotic (to be discussed later) are deposited on the paper disks by means of a micropipette having a capacity of exactly 0.005 ml. This pipette (see Fig. 1), which can be constructed easily from capillary glass tubing, is filled by capillary force after touching the solution with the open end of the pipette. Excess liquid is wiped off carefully. By touching a filter paper disk the whole content of the pipette is emptied on the filter paper by capillary forces of the fibers. Finally the moist disk is placed on a predetermined spot on the inoculated nutrient agar, by means of forceps or with the pipette, to which the disk remains adhered.

In order to use the micropipette for the next concentration or for another antibiotic solution the pipette is "rinsed" twice by allowing it to fill with this solution and emptying it by touching a piece of filter paper. Occasionally the pipette must be cleaned and degreased by placing it in warm concentrated sulfuric acid containing some nitrate.

Culture Plates.—Flat bottom Petri dishes are used with diameters of 9, 11, or 15 cm. Amounts of 16, 24, or 44 ml. nutrient agar are required in order to give a uniform layer-thickness of 2.5 mm. Care should be taken that Petri dishes during pouring and cooling are placed on a perfectly horizontal table or plate. The surface of the nutrient agar is dried before use by placing the dishes in an 37° C. incubator for a few hours.

Five or six moistened paper disks may be used for each dish.

Medium and Inoculation Procedure.—As nutrient agar a 5 per cent blood broth agar (e.g., Difco Blood Agar Base) or Levinthal agar is used (the latter for *Hemophilus influenzae*).

The infective material (pus, sputum, blood, exudate, or urine) may be streaked over the surface of the agar by means of a sterile glass rod, a wire, or a loop. However, more uniform results are obtained by using a cotton swab.

Since extremely heavy inoculation of the surface of the agar frequently gives rise to difficulties in the evaluation of results, it is advisable to make an additional dilution of the infective material in all cases where microscopic observation indicates a heavy bacterial population. For a good test it is preferable to have a large number of isolated colonies present, giving the plate a coarse grainy appearance.

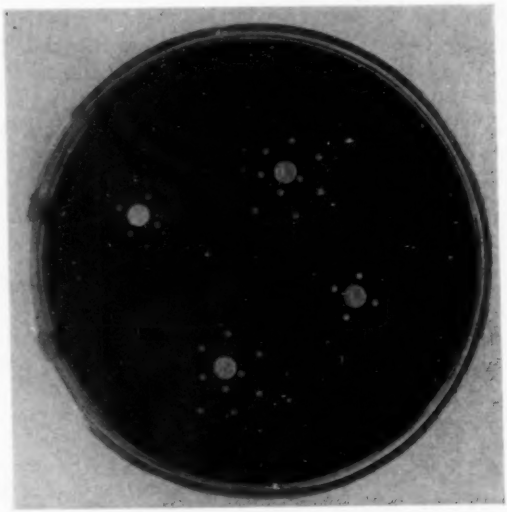


Fig. 2*A*.—Uninoculated blood plate with 4 disks each containing a predetermined amount of an antibiotic. Microcuts have been removed at different distances from the center for determination of antibiotic concentration (see Fig. 2*B*).

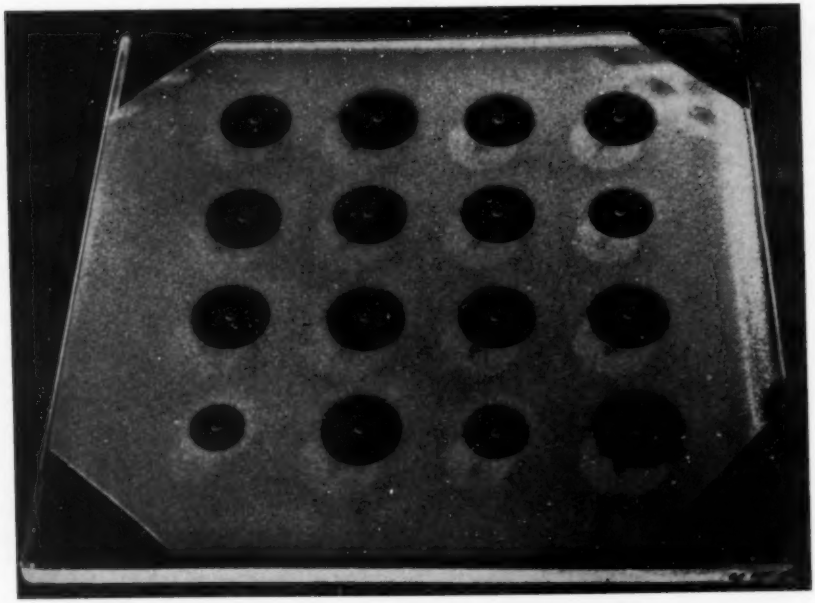


Fig. 2*B*.—Testplate with microcuts (see Fig. 2*A*) for the quantitative determination of the antibiotic in the cuts, using identical cuts with standard concentration as references. Testplate is inoculated with *B. subtilis*.

Incubation and Evaluation.—Plates are incubated for 18 hours at 37° C. Zone size is determined by the usual methods. Sometimes one may observe single colonies within the zone of inhibition. This may indicate presence of resistant variants in the infective material in addition to sensitive species. It also may indicate contamination with nonpathogenic strains. In any case, such a phenomenon should be carefully noticed and properly interpreted.

In case of swarming (e.g., Proteus strains), it is advisable to repeat the test, with blood agar plates containing 0.06 per cent sterile chloral hydrate. Another useful technique to prevent swarming is placing in the cover of the dish a piece of filter paper moistened with 10 drops of ethanol (Kellner[8]). As will be discussed below only antibiotics giving a zone size of 10 mm. or more are considered suitable for antibiotic therapy.

Diffusion of Antibiotics in Agar.—When a paper disk containing a certain amount of antibiotic is deposited on inoculated agar, diffusion takes place away from the spot of application. The concentration of antibiotic around the disk changes constantly with time. It is entirely possible that at a certain distance from the place of application of the antibiotic the bacteria are engulfed by a concentration of antibiotic sufficiently high to inhibit growth, but that at a later date this concentration decreases and becomes insufficient for inhibition. Whether at this point actually destruction of the inoculum has occurred will depend largely on the moment this suboptimal wave passes the organism. If it happens while the organism is still dormant, it might do little harm; however, when the organism is actively multiplying the effect may be lethal.

It was of interest, therefore, to study the actual antibiotic concentrations in highly standardized blood agar plates during the period of diffusion of an antibiotic out of a disk. Concentrations on increasing distance from the point of application of the antibiotic were determined. These determinations were repeated at various times after the antibiotic was allowed to diffuse away from the point of application.

In order to accomplish this the following technique was used.

Paper disks, impregnated with a known amount of antibiotic (e.g., 0.6 unit penicillin), are placed on an uninoculated blood agar plate and preincubated at 37° C. Penicillin is then allowed to diffuse freely into the agar for a certain time, e.g., 2 hours, at a temperature of 37° C. At that time we had to determine the concentration of the antibiotic at various distances from the center of the disk.

In order to determine this, we removed tiny cylindrical samples of agar at predetermined distances from the center of the disk. This was done by a method first described by Klein.[9] A Pasteur pipette* with a smooth edge was used to punch out tiny cylinders (see Fig. 2*A*).

The amount of antibiotic present in these microcuts was then determined quantitatively using identical cylindrical samples as "standards." The latter, however, were punched from similar plates which were poured separately and which contained a known concentration of the antibiotic throughout.

Testplates used for determining the antibiotic concentration were prepared according to the method used for the quantitative determination of antibiotics (Grove and Randall[10]).

The known standard, however, was not applied in the usual way by means of disks or cups on the inoculated agar layer but rather as microcuts, each containing known concentrations of the antibiotic.

After incubation of the testplates (see Fig. 2*B*) the zone size is measured and the antibiotic concentration in the unknown cylinders calculated by the usual method, based on the zone sizes produced by the standard cylinders.

By variation of test organism, size of Pasteur pipette, and thickness of the agar of the testplates, one may vary the sensitivity of the test and thus make it suitable for determination of almost any antibiotic concentration encountered in the microcuts.

*The pipette diameter is 0.75 to 2.5 mm. depending on the sensitivity desired. The cylindrical cut is blown carefully out of the pipette and deposited in vertical position on the test agar. Different predetermined distances from the center of the paper disks are marked on the agar surface by means of a pair of dividers. These imprints then serve as center for the cylinder to be removed by Pasteur pipette.

RESULTS

This method enabled us to determine quantitatively the antibiotic concentration at any distance around the disks at different times of diffusion (from 2 to 24 hours) for several antibiotics and for various concentrations of these antibiotics. The concentration of antibiotic found in the microcuts was plotted against the diameter from the zone of which the microcut formed the perimeter.

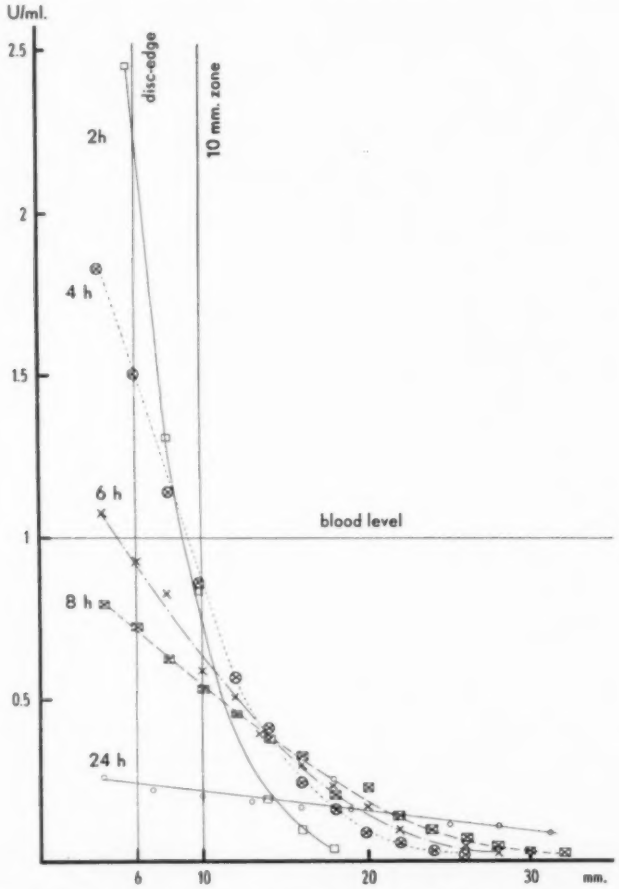


Fig. 3.—Diffusion curves with disk type Bl provided with 0.6 unit penicillin (120 U./ml.).

Thus a curve was obtained giving the antibiotic concentration at any fixed distance from the center of the disk at a definite diffusion time. By determining such curves at varying times after application of the antibiotic, an excellent impression was obtained of antibiotic concentration in the agar at any distance around the center of the disk, relative to the time over which diffusion was allowed.

Shown graphically in Figs. 3, 4, 5, and 6, respectively, are the results obtained with diffusion from disks moistened with 0.005 ml. of a solution containing 120 U./ml. penicillin, 1,000 U./ml. penicillin, 1,500 μg/ml. streptomycin, and 6,000 μg/ml. streptomycin.

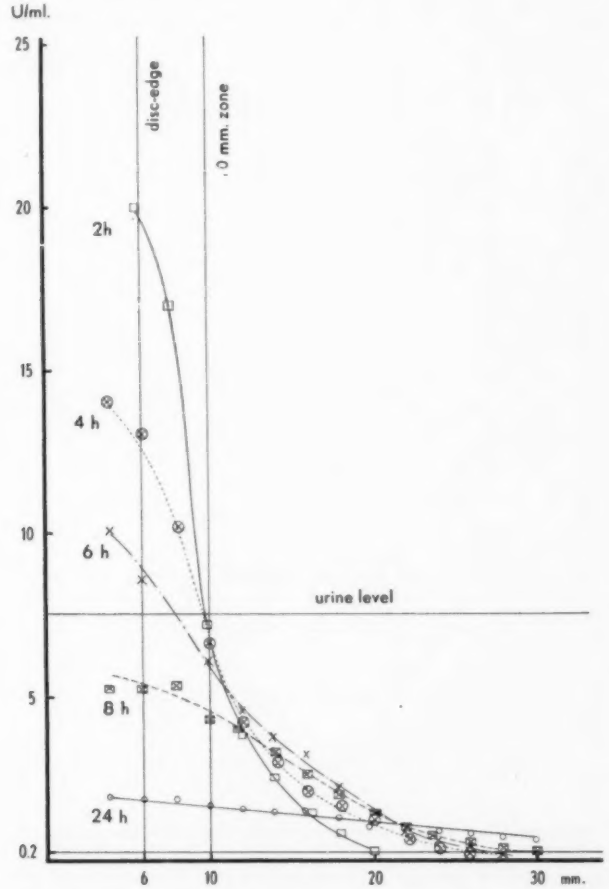


Fig. 4.—Diffusion curves with disk type Ur provided with 5 U. penicillin (1,000 U./ml.).

It may be seen from these curves that at distances of less than 5 mm. from the center (zone size $<$ 10 mm.) concentrations vary widely depending on the time allowed for diffusion. The same is true for distances beyond the 10 mm. zone size. However, there is an area around the 10 mm. zone where the concentration of antibiotic is fairly constant regardless of the time of diffusion, at least over a period from 2 to 8 hours after application of the antibiotic to the blood agar plate.

By varying the concentration of the antibiotic on the paper disk one may now produce any predetermined antibiotic concentration at the 10 mm. zone size.

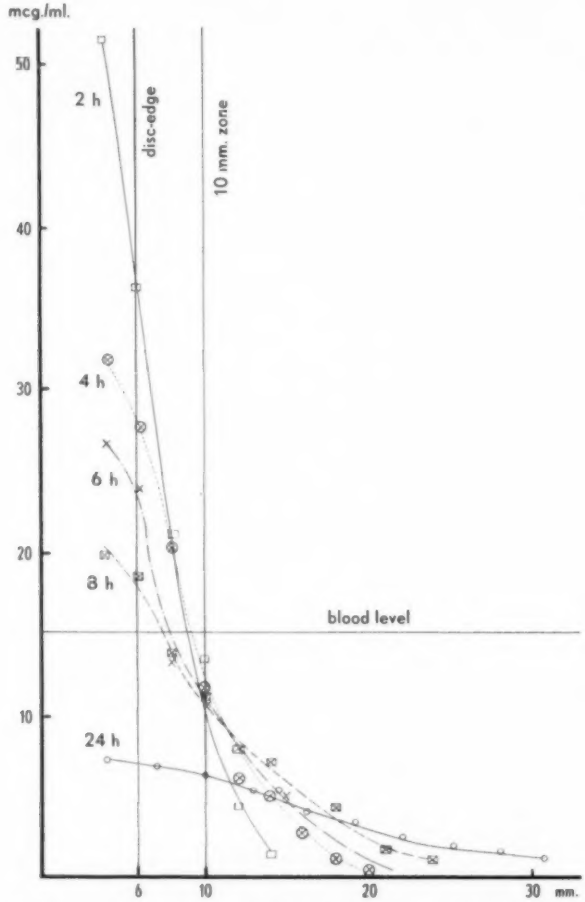


Fig. 5.—Diffusion curves with disk type B1 provided with 7.5 μg streptomycin (1,500 μg/ml.).

This relation was determined for a number of antibioties. The results of these tests are given in Figs. 7, 8, 9, and 10 for penicillin, streptomycin, chloramphenicol, and oxytetracycline. As can be seen from the illustrations there is a linear correlation between the antibiotic concentration applied on the paper disk and the concentration of the antibiotic at the 10 mm. zone.

In order to apply above results directly to the development of a method suitable for clinical application it would be desirable to produce such a concentration of antibiotic as could easily be obtained in blood (or urine) during

the course of treatment of a patient with a normal average dosage of the antibiotic at the 10 mm. zone. Such concentrations, taken from clinical literature, may be seen from Table I, column 4.

The amount of antibiotic to be deposited on a disk in order to give the afore-mentioned concentrations at the 10 mm. zone level was determined and the results of these investigations are also given in Table I, column 5.

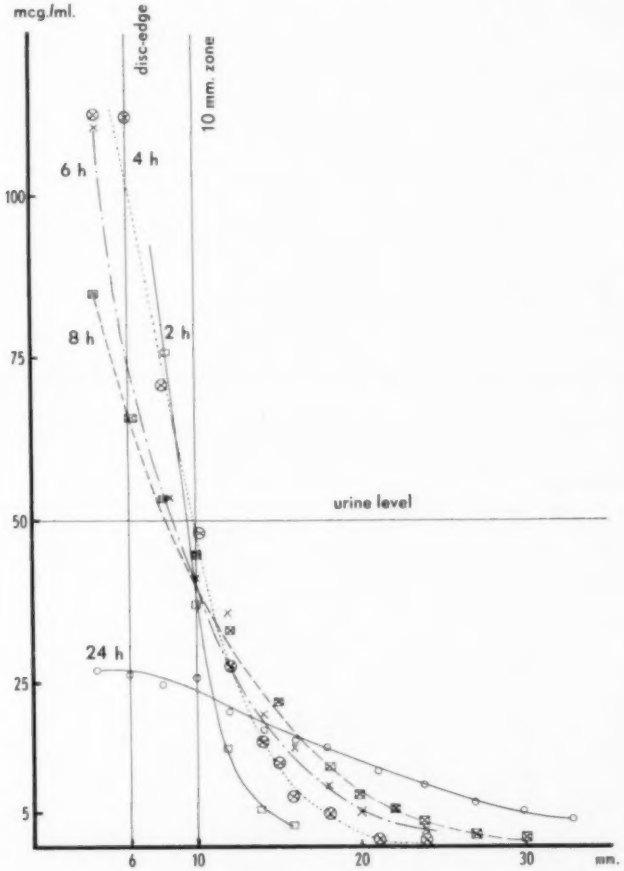


Fig. 6.—Diffusion curves with disk Ur provided with 30 μg streptomycin (6,000 μg/ml.).

As may be seen from Table I, if one applies an amount of antibiotic as listed in column 3 to the paper disks, the concentrations obtained at the 10 mm. zone limit are of the same order as can be produced by treating a patient with a normal dose schedule of this antibiotic. As pointed out already these concentrations vary little over a diffusion period from 2 to 8 hours after applica-

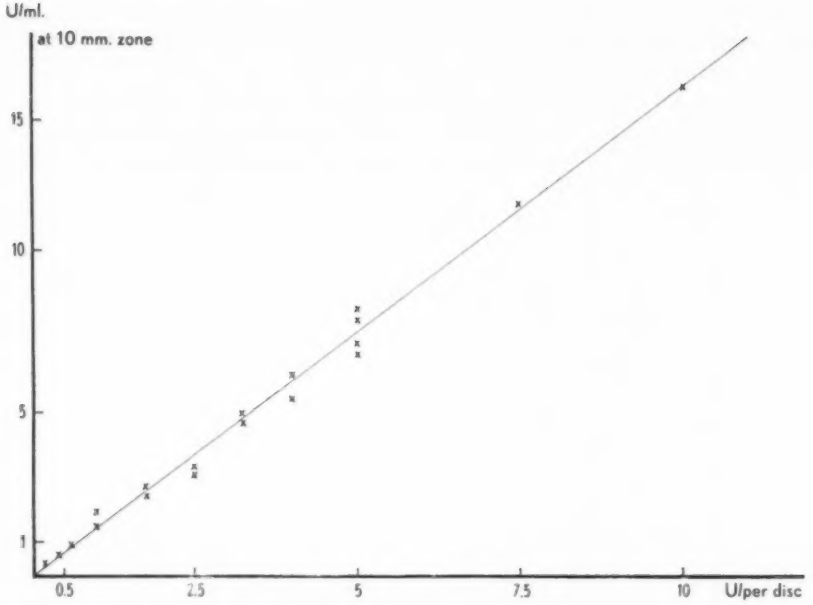


Fig. 7.—Interrelation between amount of penicillin on disk and concentration at 10 mm. zone after 5 hours of diffusion.

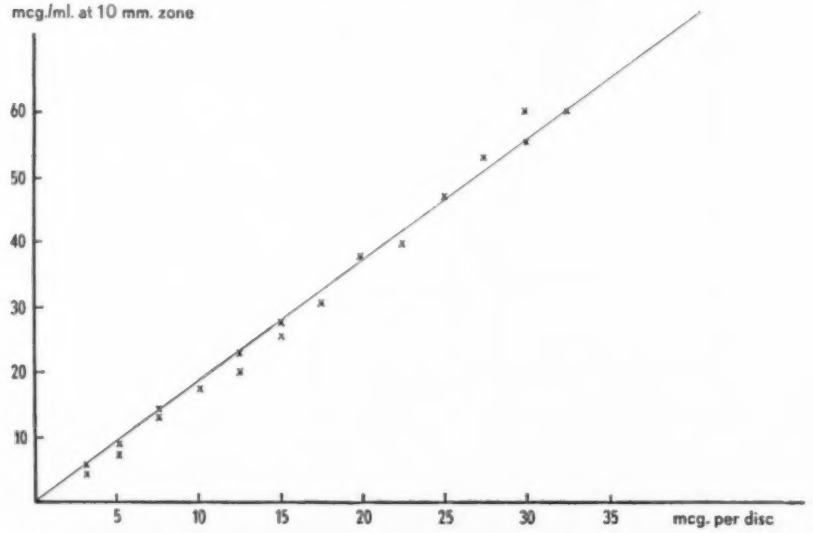


Fig. 8.—Interrelation between amount of streptomycin on disk and concentration at 10 mm. zone after 5 hours of diffusion.

tion of the antibiotic, in other words, over the entire critical period in which the ultimate zone size is fixed. Thus the error due to variations in growth rate of different species of microorganisms on zone size is largely eliminated.

If zone size for a given organism is larger than 10 mm. it implies that this organism is inhibited by antibiotic concentrations smaller than can be

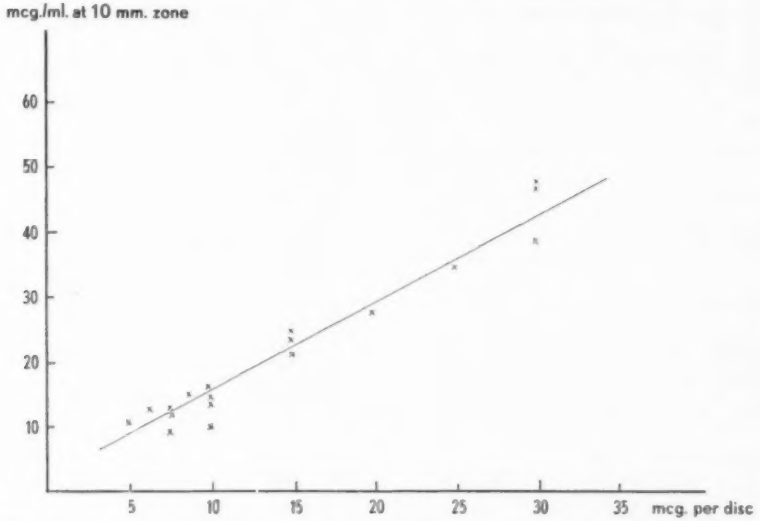


Fig. 9.—Interrelation between amount of chloramphenicol on disk and concentration at 10 mm. zone after 5 hours of diffusion.

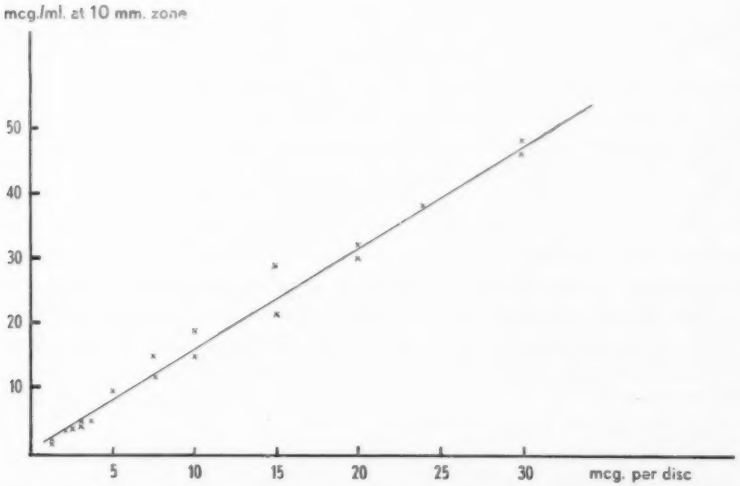


Fig. 10.—Interrelation between amount of oxytetracycline on disk and concentration at 10 mm. zone after 5 hours of diffusion.

obtained clinically. If zone size is smaller than 10 mm. the clinically obtainable blood level is insufficient to inhibit this organism.

There is, however, one important exception to this rule. Staphylococcus strains which produce penicillinase in appreciable quantities may temporarily be inhibited by concentrations of approximately 1 unit, but because of enzymatic destruction of penicillin such concentrations cannot be maintained. Sensitivity of staphylococci, therefore, presents an entirely different problem.

TABLE I. CONCENTRATION OF ANTIBIOTIC TO BE APPLIED TO PAPER DISKS (WHATMAN NO. 3) IN ORDER TO GIVE CONCENTRATIONS AT THE 10 MM. DIFFUSION ZONE COMPARABLE TO THOSE CLINICALLY OBTAINABLE IN BLOOD OR URINE (ALSO THOSE ACTUALLY DETERMINED)

ANTIBIOTIC	DISK TYPE*	AMOUNT OF ANTIBIOTIC ON DISK REQUIRED TO GIVE THE DESIRED CONC. AT THE 10 MM. ZONE	CLINICALLY OBTAINABLE BLOOD (URINE) LEVEL (= DESIRED CONC. AT THE 10 MM. ZONE)	CONCENTRATION FOUND AT 10 MM. ZONE AFTER DIFFUSION FOR: (HOURS)			
				2	4	6	8
Penicillin	Bl	0.08 U.	0.1 U.	0.14	0.12	0.11	0.11
	Bl	0.6 U.	1 U.	0.98	0.94	0.79	0.72
	Ur	5.0 U.	7.5 U.	7.2	7.5	6.7	5.0
Streptomycin	Bl	7.5 μg	15 μg	10.0	13.4	13.0	12.4
	Ur	30.0 μg	50 μg	37	50	48	46
Chloramphenicol	Bl	10.0 μg	15 μg	16.0	15.3	12.4	10.9
	Ur	30.0 μg	50 μg	62	57	47	42
Oxytatracycline	Bl	3.0 μg	5 μg		4.4	4.5	3.7
	Ur	30.0 μg	50 μg	44	50	51	49
Tetracycline	Bl	3.0 μg	5 μg		5.2	4.5	4.2
	Ur	35.0 μg	50 μg	41	47	48	53
Erythromycin	Bl	3.0 μg	5 μg		4.9	5.2	3.8
	Ur	30.0 μg	50 μg		52	46	42
Bacitracin	Bl	0.6 U.	1 U.		0.99	0.82	0.78
	Ur	18.0 U.	30 U.		28	28	28
Neomycin	Bl	0.6 U.	1 U.		1.0	1.0	0.97
	Ur	30.0 U.	50 U.		51	47	50

*Bl = blood; Ur = urine.

From practical experience it was concluded that in order to treat staphylococcus infections effectively with penicillin we should restrict ourselves to the nonpenicillinase producing strains of staphylococci. In general such strains are sensitive to < 0.1 unit penicillin. For this reason a special penicillin concentration is applied giving a 0.1 unit penicillin concentration at the 10 mm. zone limits, instead of the usual 1 unit for blood plates.

In case we are dealing with staphylococci, the zone size around these special disks should be in excess of 10 mm. before recommendation for the treatment of such staphylococcus infections with penicillin is advocated.

Comparison of the Modified Disk Method With the Dilution Method.—A comparative study was made of the relationship between the results of the sensitivity of an organism for an antibiotic as determined by dilution method and the sensitivity as determined by the modified disk method, using the observed results at the 10 mm. zone as criteria. A large number of strains from divergent species was investigated.

In all cases where the results of dilution test were within range of those found by the disk method, correlation was considered "good" ("g" in columns of Table II). For instance if the dilution method indicated that the sensitivity

of an *Esch. coli* strain against streptomycin is 7.5 μg/ml., the inhibition zone on a Bl plate must be *in excess of* 10 mm. since at the 10 mm. zone limit a streptomycin concentration of approximately 15 μg is present.

In case no zone would be observed, correlation would be considered "poor" ("p").

In a case the zone size would be about 8 mm. or 12 mm. instead of an expected zone size of 10 mm., correlation would be considered "fair" ("f").

Table II shows that for 4 antibiotics and a total number of 466 tests, in 97 per cent of the cases a good correlation was obtained between the two methods.

TABLE II. CORRELATION BETWEEN THE SENSITIVITIES ACCORDING TO THE DISK METHOD AND THE DILUTION METHOD

	TOTAL NUMBER OF ASSAYS	PENICILLIN			STREPTOMYCIN			CHLORAMPHENICOL			OXYTETRACYCLINE		
		G	F	P	G	F	P	G	F	P	G	F	P
Staphylococci	114	15			36	1		31	1		30		
Enterococci	21	4			6	1		4			6		
H. influenzae	17	12	2	1							2		
Escherichia	50	6			11			12			21		
Pseudomonas	120	20			32	1	1	28			34	2	2
Salmonella	29	6			7	1	1	6			8		
Proteus	64	14			15	1	1	16			17		
Various gram neg.	51	14			13			12			12		
	466	91	2	1	120	5	3	109	1		130	2	2

Total number of assays	466
good correlation	450
fair correlation	10
poor correlation	6

Key: g = number of assays with good correlation; f = number of assays with fair correlation; p = number of assays with poor correlation.

On the basis of these results we believe that the modified disk method gives a more accurate evaluation of the sensitivity of pathogenic microorganisms for antibiotics than the usual diffusion method with disks, which contain in general much larger quantities of antibiotic. It must be realized that in vitro determinations do not always correlate entirely with in vivo results, but this shortcoming is inherent to all laboratory determinations of sensitivity. However, practical experience has proved conclusively the usefulness of in vitro laboratory determinations to serve as a guide for the selection of the correct antibiotic in the treatment of infectious diseases.[11-12]

DISCUSSION

It has been pointed out that the selection of antibiotic concentrations at the 10 mm. zone is directly correlated with literature data on obtainable blood or urine levels in patients treated with a "normal" dosage scheme of an antibiotic. In case clinical correlation proved to be inadequate the concentration deposited on the disk can easily be altered to correct such a possible discrepancy.

Also for veterinary use the amount of antibiotic deposited on the disk can be adjusted to levels adapted to the customary treatment of animals with antibiotics.

Finally we want to point out the reasons why direct smears of infective material were preferred over those of pure cultures.

Apart from considerable loss of time necessary to isolate such a pure culture from clinical material there are certain other pertinent disadvantages to this method.

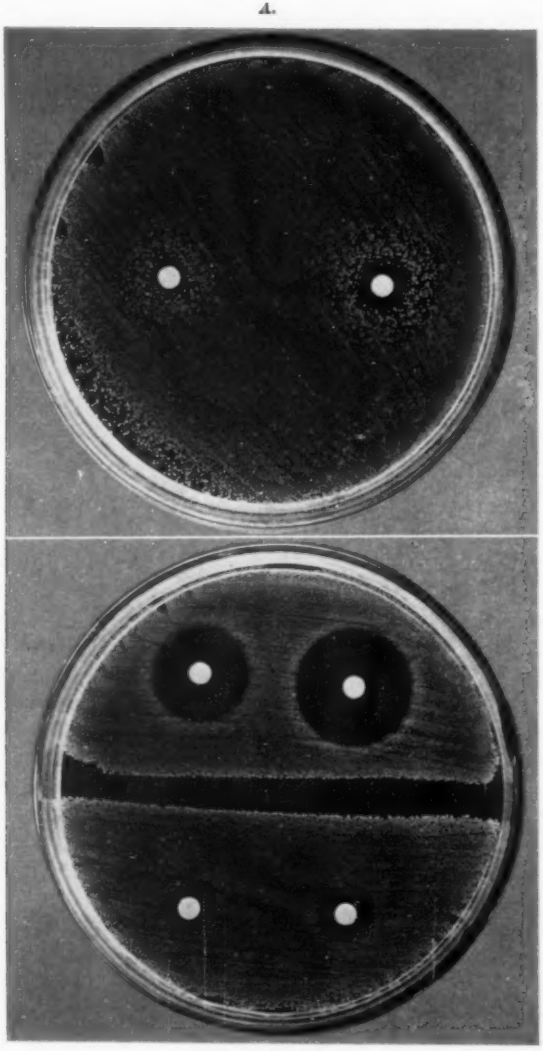


Fig. 11.—*A*, Direct smear from a *Staph. aureus* infection with Bl and Ur type disks, containing chloramphenicol. One may observe many resistant colonies in the inhibition zones. *B*, (Above) Secondary smear from a colony taken at random from plate *A*, tested against chloramphenicol (Bl and Ur type disks). Result: sensitive. (Below) Secondary smear from a colony taken from the inhibition zone from plate *A*, tested against chloramphenicol (Bl and Ur type disks). Result: resistant.

1. Although infective material may contain only a single species of a pathogen it is entirely possible that the population is not uniform in regard to sensitivity to the antibiotic. This may be due to the fact that we are dealing with a mixed infection of two or more strains of the same species, but with naturally different sensitivity; or it may be due to the presence of naturally resistant mutants of the same strain. When first a pure culture is isolated, the progeny of one single cell, which might not represent the average sensitivity of the total population, is selected for the test. In fact, resistant bacteria which may be present in small numbers in the infective material may thus be overlooked. The direct smear method on the other hand readily demonstrates presence of resistant strains by yielding a number of isolated colonies directly in the zone of inhibition (see Fig. 11, *A* and *B*). When this occurs the clinical usefulness of such an antibiotic becomes problematic.

The correct evaluation of appearance of colonies in the inhibition zone is very important. In fact the appearance of the zone around the disk has definite diagnostic value. We like to refer to it as: the antibiotic-diagnostic zone. This can be seen in the following example.

Pus from a wound of a patient was smeared on blood plates for the purpose of establishing the sensitivity of the infective organism against antibiotics by the afore-mentioned modified paper disk method.

The infective organism was identified as a Proteus. No zones of inhibition appeared around the penicillin and oxytetracycline disks but there were definite (though incomplete) zones of inhibition around the streptomycin and chloramphenicol disks. Strains were isolated from the inhibition zones around the streptomycin and the chloramphenicol disks, respectively. Both were Proteus strains. However, one strain was streptomycin resistant, but chloramphenicol sensitive, whereas the other strain was streptomycin sensitive, but chloramphenicol resistant. This was not only confirmed by the modified paper disk method but the dilution method gave identical results.

Both Proteus strains occurred in about equal quantities in the pus. In case a pure culture had been isolated first, chances are that it would have been either the chloramphenicol-sensitive or the streptomycin-sensitive strain and, consequently, the wrong therapy could have been instituted with either streptomycin or chloramphenicol.

In this case the patient should have received a combined therapy with both antibiotics. According to Barber, as cited by Martin and associates,[13] such mixed infections are by no means a rarity.

2. It is also possible that mixed infections are encountered with two or more species of pathogens of different genus, e.g., a mixed Streptococcus and Proteus infection. It is always advisable to check the possibility of such mixed infections by streaking the infective material on blood-agar plates specially prepared for this purpose. In the presence of Proteus, because of its spreading properties, it is frequently difficult to detect the primary cause of the infection as is illustrated by the following example.

A urine sample of a patient was submitted for test by the modified disk method. The following day the plate was fully overgrown with a Proteus strain which appeared to be sensitive to both tetracycline and oxytetracycline. However, within the inhibition zone one could observe clearly a fairly large number of minute Streptococcus colonies. This

organism could be isolated in pure culture. The special plate prepared to check mixed infections showed no such colonies; probably because in the low dilution they were overgrown by Proteus and in high dilution were diluted out entirely.

This is another case indicating the value of the antibiotic diagnostic zone in case of mixed infections.

A note of warning must be made against the use of the so-called accumulation technique prior to determination of sensitivity by the paper disk method. This is frequently advocated in case the infective material, e.g., sputum, is very low in microorganisms. The danger inherent to this method is obvious since frequently totally unimportant contaminants may overgrow the infective pathogens.

Notwithstanding the drawback that standardizing the amount of inoculum on the testplates is not very well possible if direct smears with the infective material are used, this technique is preferable over the isolation technique.

No method, however refined it may be, can eliminate the necessity of the trained eye of the bacteriologist, and the correct evaluation of what is observed is always of prime importance.

CONCLUSION

A modified paper disk method is described by which it is possible to determine the sensitivity of microorganisms against antibiotics.

This method is based on producing at the 10 mm. zone size such a concentration of antibiotic as can be easily obtained clinically as blood level (or urine level) by the usual average antibiotic therapy. In case zone sizes of more than 10 mm. are observed under otherwise standardized conditions, the antibiotic may be advocated for clinical treatment of the patient.

For differentiating between penicillinase and nonpenicillinase producing staphylococci a special disk is introduced giving a penicillin concentration at the 10 mm. zone of 0.1 unit; zones < 10 mm. being an indication for penicillinase production.

Comparative experiments indicate that there exists a good correlation between the results obtained with the modified disk method and the dilution method.

We wish to express our thanks to Miss C. W. J. M. Calame and Miss G. Santing for valuable technical assistance. Our special thanks are due to Dr. W. R. O. Goslings and Dr. J. C. Hoogerheide for their advice and encouragement during these studies.

REFERENCES

1. Florey, H. W., Chain, E., et al.: Antibiotics, London and New York, 1949, Oxford University Press.
2. Erlanson, P.: Determination of the Sensitivity in Vitro of Bacteria to Chemotherapeutic Agents, Acta path. et microbiol. scandinav. (Suppl.) **85: 1**, 1951.
3. Gould, J. C., and Bowie, J. H.: The Determination of Bacterial Sensitivity to Antibiotics, Edinburgh M. J. **59**: 178, 1952.
4. Jackson, G. G., and Finland, M.: Comparison of Methods for Determining Sensitivity of Bacteria to Antibiotics in Vitro, Arch. Int. Med. **88**: 446, 1951.
5. Kenney, M., Johnson, P. M., and Tatz, J. S.: A Four Year Study of Bacterial Sensitivity to Five Antibiotics Using a Standardized Paper-Disc Method, Antibiotics & Chemother. **3**: 1221, 1953.

6. Schönfeld, J. K.: The Investigation of Bacterial Sensitivity in Regard to Antibiotics, Thesis, Leiden, 1953.
7. Welch, H.: Antibiotic Sensitivity Testing, Antibiotics & Chemother. **6**: 321, 1956.
8. Kellner, W.: Über ein einfaches Verfahren der Hemmung des Schwärmens von Bact. Proteus, Zentralbl. Bakt. (Abst. 1) **161**: 150, 1954.
9. Klein, P.: Über die Diffusionsgeschwindigkeit des Penicillins im Agar und ihre Interferenz mit der Latenzzeit des Inoculums bei der Resistenzprüfung, Zentralbl. Bakt. (Abst. 1) **159**: 301, 1953.
10. Grove, D. C., and Randall, W. A.: Assay Methods of Antibiotics: A Laboratory Manual, Medical Encyclopedia, New York, 1955.
11. Broom, N. H., Martineau, P. C., and Young, D. C.: A Correlation of the Results of Disk Antibiotic Sensitivity Tests and the Clinical Course of Sixty-Nine Patients With Acute Bacterial Meningitis, Antibiotics & Chemother. **3**: 409, 1953.
12. Rodger, K. C., et al.: Correlation of Clinical Results With Laboratory Sensitivity Tests, Canad. M. A. J. **74**: 605, 1956.
13. Martin, R., Chabbert, Y., Sureau, B., Véron, M., and Martin, L.: Intérêt des antibiotiques nouveaux et des données du laboratoire, Les nouveaux antibiotiques et l'orientation actuelle de l'antibiothérapie, Rapport's présentées au XXX e congrès Français de médecine, Alger, 1955, p. 92.

MODIFICATION OF THE TUBELESS BEDSIDE TEST FOR GASTRIC ACIDITY

J. M. Holthaus, M.D.,* F. L. Humoller, Ph.D.,** and J. R. Walsh, M.D.***
Omaha, Neb.

THE examination of gastric contents for the presence or absence of free hydrochloric acid has long been an accepted diagnostic procedure. Frequently the information desired from this test is of a qualitative nature and it is important only to establish the presence or absence of absolute achlorhydria. There are a number of disadvantages to the test such as the discomfort to the patient and the difficulty in passing the gastric tube. Therefore it was important to develop a quick, simple, and convenient method for screening the patients without subjecting them to gastric intubation.

In 1950, Segal and associates[1] described a simple test for the detection of gastric acidity. This test involved the administration of cation exchange resins in the quininium form and the detection of quinine fluorescence in the urine one and two hours later. In some respects this test had several advantages over the intubation method, such as the ease with which it could be performed. However, the detection and measurement of the quinine fluorescence not only involves the use of ultraviolet light and a darkroom, but also solvent extraction procedures to remove nonspecific fluorescence and substances which may have a quenching effect upon quinine. It is the purpose of the present report to describe a modification of the Segal test which eliminates these disadvantages and which is readily adaptable to bedside testing since only a few test tubes or graduated cylinders, a medicine dropper, and a few simple reagents are required.

METHOD

Reagents.—Ammonium sulfate; purified, granular. Any laboratory grade is suitable.

TBPP, 0.1 per cent. Dissolve 100 mg. of tetrabromophenolphthalein *ethyl ester*[a] in 100 ml. of absolute alcohol. Store in refrigerator.

Benzene, reagent grade.

Sodium hydroxide 4 per cent. Dissolve 4 Gm. of sodium hydroxide in 100 ml. of water. Keep in stoppered container.

From the Veterans Administration Hospital, Creighton University School of Medicine, and the University of Nebraska College of Medicine, Omaha, Neb.

Received for publication Sept. 3, 1957.

*Chief, Medical Service, Veterans Administration Hospital, Omaha, Neb., Assistant Professor of Medicine, Creighton University School of Medicine; Assistant Professor of Medicine, University of Nebraska College of Medicine.

**Principal Scientist, Medical Research Laboratory, Veterans Administration Hospital, Omaha, Neb., and Associate Research Professor, Chemical Physiology, University of Nebraska College of Medicine, Omaha, Neb.

***Director, Department of Medicine, and Professor of Medicine, Creighton University School of Medicine, Omaha, Neb.

Procedure.—

A. Preparation of patient: (1) Have the patient fast overnight. (2) The morning urine obtained on arising is discarded. Then have the patient drink ½ glass of water containing 250 mg. of caffeine sodium benzoate.† (3) One hour later collect the urine in bottle labeled "control." (4) Now have the patient drink ¼ glass of water in which 2 Gm. of quininium resin granules† are suspended. Follow-up with another ¼ glass of water. (5) One hour after taking granules have patient urinate into bottle labeled "Urine Sample 1." (6) One hour later have patient urinate into bottle labeled "Urine Sample 2."

B. Colorimetric detection of quinine in the urine: (1) Place about 10 ml. of each of the urine samples in 15 ml. graduated test tubes. (2) To each tube add a heaping teaspoonful (5 to 6 Gm.) of ammonium sulfate and shake to dissolve most of it. (3) Next add to each tube 3 drops of 0.1 per cent tetrabromophenolphthalein ethyl ester in ethanol and mix. (4) Add about 1 ml. of benzene to each tube. (5) Add 3 to 4 drops of 4 per cent NaOH and shake well. (6) Allow layers to separate for a minute or two.

C. Interpretation of the color produced: The urine sample collected before the administration of the quininium resin serves as control for the other two samples. The benzene (upper) layer should be a clear yellow without any tinge of red or orange. If a reddish color is obtained in this sample it usually means that the patient has received quinine in his medication. In such cases the test must be repeated 2 or 3 days after all quinine medication has been stopped.

In the achlorhydric patient the second and third urine samples will have the same clear yellow color as the control sample. In borderline cases, that is, in cases with slight free acidity the control sample will be quite yellow, but either the first or second sample, or both, will show a definite orange to orange red tinge. In this laboratory such response is evaluated as 1+ or 2+. In the normal or hyperacidic patient the control sample will be yellow, but the subsequent samples will show a bright cherry-red color in the benzene layer. Such a response is recorded as either 3+ or 4+, depending upon the intensity of the color.

RESULTS

This test has now been performed on more than 100 patients, including 11 patients with pernicious anemia, 78 medical students, and a number of cases with varying clinical conditions. These cases, with only a few exceptions, were also given the standard gastric analysis test with the aspiration technique. In the 78 medical students, all presumably with normal gastric acidity, the test was positive in 77 of the cases and negative in one individual who did show gastric acidity when tested by the aspiration technique. On repeating the proposed test a trace of acid was found. This particular individual has had many gastrointestinal complaints including epigastric distress, intermittent episodes of slight anorexia and nausea, and some gaseous formation. On two separate occasions GI series demonstrated a very rapid emptying time of the stomach with no barium remaining in the stomach after one hour. No other pathologic lesions were noted.

All 11 cases of pernicious anemia showed absolute achlorhydria with both methods. Of the remaining cases with varying clinical conditions, most of which were studied with both the aspiration and the tubeless methods there were corresponding results in all patients but one. In this case free HCl was

*Eastman Kodak Company, No. 6810.

†Commercially available in the form of Diagnex test package, quininium formula. E. R. Squibb & Sons, New York, N. Y.

found by the proposed test and achlorhydria by the aspiration method. Unfortunately this could not be rechecked. In one other patient, similar results were obtained on the first testing, but, on retesting, free HCl was found with both methods, therefore confirming the initial results of the proposed test. In another patient where quantitative comparisons were made, the aspiration technique showed 48° of free HCl and the proposed test revealed only a trace of acid. On checking it was found that the patient had severe renal insufficiency. This perhaps explains the appearance of only a small amount of quinine in the urine. Because of this, two other patients with severe renal insufficiency were tested with the tubeless method and both were found to have small amounts of acid by this test. A few patients with hepatic insufficiency were tested, and contrary to what was expected, these patients also showed only a trace of acid by the tubeless method. In all other cases comparable results were obtained by the old standard method and the presently proposed test.

DISCUSSION

The present studies were performed using Diagnex which is essentially a carboxylic type of cation exchange resin in the quininium form. The resin has a greater affinity for hydrogen ions than for quininium ions and upon reaching the stomach quinine is released and absorbed into the blood stream in proportion to the acidity of the gastric juice. Once absorbed a variable portion (60 to 90 per cent)[2] of it is destroyed in the body while the rest is excreted in the urine. Hence, in cases of liver or kidney dysfunction the results obtained by the test must be interpreted with some caution.

In the proposed test quinine is detected by utilizing its properties of an organic base. If certain acid dyes are added to urine containing quinine, a quinine salt of the dye is formed which is insoluble in water but soluble in benzene, thus forming the basis for a sensitive test for quinine in urine. Tetrabromophenolphthalein ethyl ester (but not the free acid) is only one of several dyes which can be used for this purpose but our studies indicate that it is the most convenient and sensitive dye for this test.

In carrying out the procedure all urine samples are saturated with ammonium sulfate to precipitate any protein that might be present. The addition of the ammonium sulfate to the urine also increases the density gradient between the organic and the aqueous phases and brings about quicker and sharper separation of the two layers without centrifugation. Furthermore this high salt concentration reduces the solubility of the dye-quininium complex in the aqueous phase and thus increases the sensitivity of the test.

The main advantage of the test lies in its ability to separate quickly and conveniently achlorhydric patients from those who still produce hydrochloric acid. If a quantitative interpretation of the results of the test is to be made it should be kept in mind that the amount of quinine destroyed in the body may vary from paient to patient. The total amount of urine excreted during the test may also influence the results somewhat. Renal insufficiency may give a false negative result, whereas hepatic insufficiency may be expected to give

a false positive in some instances. Although the gastric intubation test, if properly carried out, yields quantitative data, it likewise is subject to several sources of errors such as faulty placement of the tube, aspiration of mucus, psychic suppression of the gastric secretion, and a measurement of hydrochloric acid concentration rather than total amount. The tubeless method yields only qualtitative results, or, at best roughly quantitative but it has the advantage of being a simple test and eliminates the discomfort to the patient.

In clinical medicine both the tubeless and intubation method should find their place. The tubeless method should be used to screen out the definitely positive or negative reacting patient. The intubation method can be used in conjunction with the tubeless method to study the borderline cases.

While these studies were in progress Bolt and his co-workers[3] reported the results of a study with a new formula Diagnex incorporating a dye with a cation exchange resin, which, when taken by mouth in the presence of free hydrochloric acid in the stomach, releases the dye into the blood stream and the dye is later recovered in the urine. Detection of the dye in the urine, however, in some cases requires that the urine be boiled for 10 minutes and allowed to stand for 2 hours. The toxicity of the dye awaits further studies. The presently proposed test eliminates these two factors and therefore it is felt to be superior.

SUMMARY

A preliminary report of a new modification of the tubeless gastric analysis test has been presented. It is a simple and qualitative test which is convenient, and can be performed as an office procedure without the aid of ultraviolet light or darkroom. The test should not be considered as a substitute for the quantitative intubation method, but rather as an adjunct to it in that it permits the screening of a number of patients with comparative ease and little discomfort to the patients.

REFERENCES

1. Segal, Harry L., Miller, Leon L., and Morton, John J.: Determination of Gastric Acidity Without Intubation by Use of Cation Exchange Indicator Compounds, Proc. Soc. Exper. Biol. & Med. **74**: 218, 1950.
Sievers, M. L., and Gielselman, R. V.: Tubeless Gastric Analysis, Am. J. Digest. Dis. **1**: 241, 1956.
2. Goodman, L., and Gilman, A.: The Pharmacological Basis of Therapeutics, New York, 1941, The Macmillan Co., p. 910.
3. Bolt, Robert J., Ossius, Theodore G., and Pollard, H. Marvin: A Clinical Evaluation of Tubeless Gastric Analysis, Gastroenterology **32**: 34, 1957.

A MODEL SPOTTING APPARATUS FOR THE AUTOMATIC DEPOSITION, ON FILTER PAPER, OF SOLUTIONS FOR CHROMATOGRAPHIC SEPARATIONS

JOHN LOGOTHETIS, M.D., AND FREDERICK SHERMAN*
MINNEAPOLIS, MINN.

FOLLOWING the original work in paper chromatography of Consden, Gordon, and Martin,[1] this method has become the procedure of choice for the separation of various substances in mixtures and biologic fluids. Despite numerous modifications this technique has remained virtually unchanged since the original description.

During the last 5 years we have been engaged in the chromatographic analysis of free amino acids in the cerebrospinal fluid. Soon after our study began it was apparent that the tedious manual spotting of this biologic fluid on the filter paper was the main limiting factor in our work. As the concentration of amino acids in the cerebrospinal fluid is some ten times smaller than in plasma, it was found necessary to spot at least 1 ml. for satisfactory results. For such a volume some 400 manual applications were required if a good small deposition spot was to be achieved. Concentration of the sample was tried in order to eliminate partially this time-consuming factor. A final volume of at least 0.25 ml., however, was still required in order to handle properly the manual spotting. It is obvious that similar problems may arise in any chromatographic work of biologic fluids, tissue extracts, or other mixtures if the substances under investigation are present in minute concentrations. Moreover, the limitations in the spotting of such fluids become greater in dealing with substances of low solubility as they may not go into solution when the volume of the original specimen is markedly reduced. In addition, significant solutes in a small concentrated specimen may be wasted by deposition on the inner surface of the container tube.

In order to facilitate spotting and to avoid concentration of the specimens, a spotting apparatus was designed and constructed allowing simultaneous automatic application of the samples. This apparatus has been extensively used in our study of free amino acids in the cerebrospinal fluid.[2-4]

Description of the Apparatus.—The spotting apparatus is demonstrated in Fig. 1. The essential mechanics involved in this apparatus can be studied in Fig. 2. An electric step-down motor (A) "Bodine CR-2" of gear ratio 289-1

From the Division of Neurology, University of Minnesota Medical School, Minneapolis, Minn.

This investigation aided by grant number B-1183 from the National Institute of Neurological Diseases and Blindness, National Institutes of Health.

Received for publication Sept. 11, 1957.

*Present address: 2526 College Avenue, Berkeley, Calif.

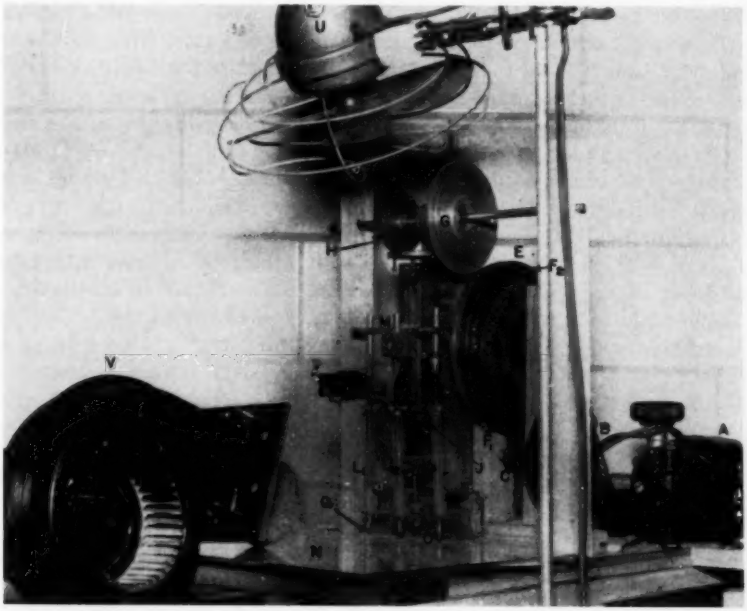


Fig. 1.—The spotting apparatus in the present stage of development. (See text for explanation.)

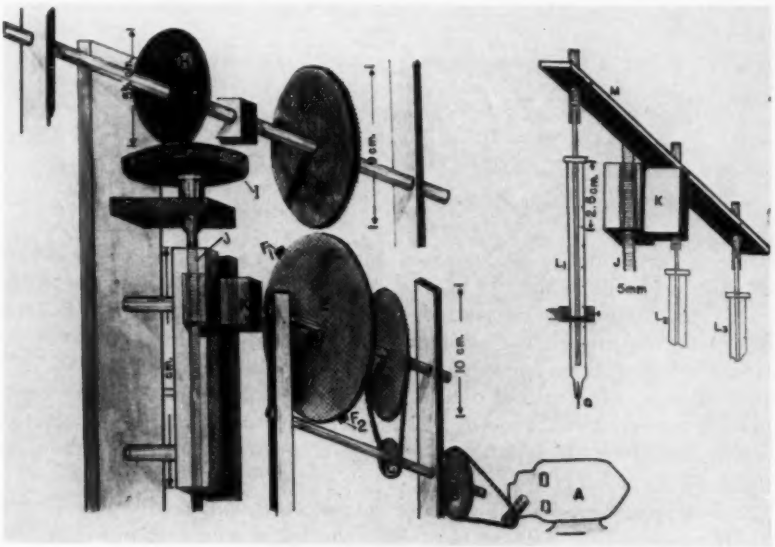


Fig. 2.—The essential parts of the spotting apparatus. (See text for explanation.)

connected to gear train (B, C, and D) turns a disk (E), while two sets of cogs (F_1 and F_2) on the disk (E) periodically engage gear (G). The turning of the latter gear rotates the disks (H and I). The two latter disks at right angles to each other make a rubber-to-rubber contact. The disk (I) is attached to a threaded rod (J) contacting corresponding threads of the sliding block (K). Upon the latter block there is attached a carriage bar (M) (in Fig. 2 this connection is shown separately) which holds the plungers of 3 tuberculin syringes (L1, L2, and L3) containing the solution under investigation. With the entire unit in operation the periodic engagement of the cogs (F_1 and F_2) with the gear (G) results in an intermittent downward movement of the sliding block (K) carrying the bar (M). The plungers attached to bar (M) move within the syringes which are firmly mounted on the frame of the device. This periodic movement of the plungers results in the discharge of a constant size drop of fluid from each syringe which is deposited on filter paper.

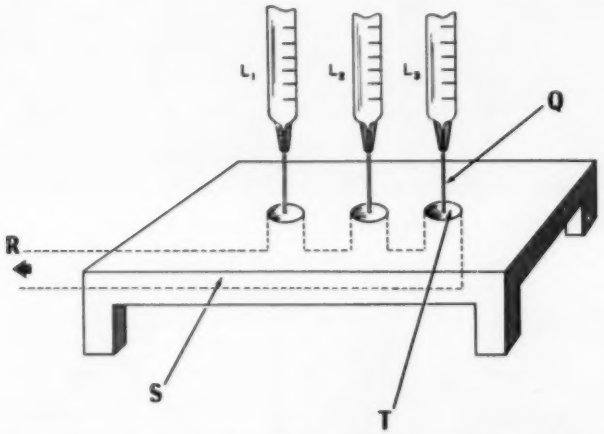


Fig. 3.—The connections of the suction drying system mounted beneath the table of the spotting apparatus. (See text for explanation.)

The size of the drop may be increased or decreased if necessary by changing the number of teeth in the two sets of cogs on the disk (E), thus controlling the delivered volume by varying the degree of rotation of gear (G) which regulates the downward movement of the plungers. A finer control of the drop size is achieved by loosening temporarily and sliding the disk (H) on its supporting axis. The rate of delivery of the drops is adjusted by a rheostat regulating the speed of the motor. The latter adjustment allows for variations in the drying time which is dependent upon the temperature and humidity of the room.

Each drop is deposited on the filter paper which is placed in contact with the delivering part of each syringe. This contact is seen only in Fig. 1. The filter papers are placed on the mounting table of the machine and are covered by a piece of plate glass (N) designed to afford protection against dirt while

leaving only a small area (O) for spotting of the papers. Contact between each syringe with the paper is made by means of a small section of 1 ml. bore polyethylene tubing (Q) rather than a steel needle. (See Figs. 1 and 3.)

Drying between each deposition of the drops on the paper is accelerated by reducing the vapor pressure under each wet spot. This is achieved by means of a suction system opening into circular holes on the mounting table and corresponding to the areas of the papers containing the wet spots under each syringe. This is seen in Fig. 3 where suction (R) is applied through a closed tube system (S) to openings (T) centered below the syringes. Drying can also be further accelerated by forced air from a fan (U) or blower (V) as shown in Fig. 1. It is best, however, to avoid forced air in order to prevent the deposition of any dust on the wet spots. If suction alone is used, the spotting of 1 ml. of solution in water requires approximately 8 hours. This time is considerably reduced when both suction and forced air are applied. It is advisable to add some preservative to the solution within each syringe for protective purposes.

After complete delivery of the samples the apparatus automatically cuts off when contact is made between the carriage bar (M) and a small electric microswitch (X) seen only in Fig. 1.

This apparatus represents the original design and construction. A unit mechanically improved to handle up to 8 delivering units is now under development.

SUMMARY

1. An automatic spotting apparatus for the use in paper chromatography has been described.

2. This unit renders unnecessary the concentration of specimens spotted to the chromatographic paper.

REFERENCES

1. Consden, R., Gordon, A. H., and Martin, A. J. P.: Qualitative Analysis of Proteins. A Partition Chromatographic Method Using Paper, Biochem. J. **38**: 224, 1944.
2. Logothetis, J.: A Study of Free Amino Acids in the Human Cerebrospinal Fluid, Neurology **5**: 767, 1955.
3. Logothetis, J.: The Free Amino Acid Content of Cerebrospinal Fluid in Humans and Dogs, Neurology. (In press.)
4. Logothetis, J.: A Study of Cerebrospinal Fluid Free Amino Acids in Neurological Diseases. Observations in Aspartic Acid Concentrations, Neurology. (In press.)

A METHOD OF HOLDING MICE FOR INTRAVENOUS INJECTION

NED FEDER, M.D.
BETHESDA, MD.

A MOUSE must be immobilized during intravenous injection through a tail vein. Usually the mouse is held by an assistant or is introduced snugly into a tube from which the tail protrudes. The method to be described here allows one to inject easily and rapidly without an assistant. With practice one can inject 50 to 100 mice per hour. The method lends itself especially to the safe injection of infectious material. The mouse holder has probably been devised independently by others, but interest shown in the technique by several visitors to our laboratory prompts this description.

A plastic mouse cage (ours is $\frac{1}{8}$" thick and 6" deep by 10" by 20") is slotted at one end with a straight vertical slot open at the top and running from the top edge to a point about $\frac{1}{2}$ inch from the bottom of the cage (Fig. 1). The slot is sawed out so as to taper from a width of about $\frac{1}{2}$ inch at the top to about $\frac{1}{8}$ inch at the bottom. The bottom of the slot is smoothed with a round file. The box may be weighted to keep it from sliding.

A mouse is seized by the tail and lowered along the slot until it is standing on the floor of the box. The tail passes through the slot to the outside, where the tip of the tail is still held in the experimenter's fingers. The experimenter pulls the tail taut with one hand, pulling the mouse's trunk tightly against the slotted wall of the cage, and keeping the tail straight and immobile. With his other hand the experimenter may now insert a $\frac{1}{2}$ or $\frac{3}{4}$ inch, 27 gauge needle into a lateral tail vein and inject fluid intravenously. After injection of infectious material a drop of tincture of iodine is placed on the injection site.

Any of the usual methods may be used to dilate the vein before inserting the needle, though these usually lead to increased bleeding after the needle is withdrawn. The mice may be kept in a hot room just before injection. The tail may be heated or moistened with benzene or xylene. Often simply rubbing the tail vigorously with the fingers dilates the vein sufficiently.

From the U. S. Department of Health, Education, Welfare, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

Received for publication Sept. 14, 1957.

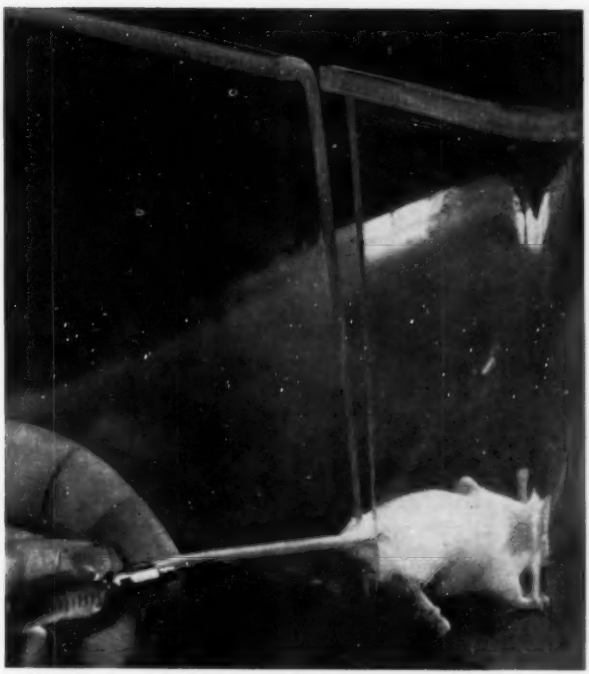


Fig. 1.—Demonstration of method of holding mice in plastic cage. Mouse shown in position for injection.

PROCEDURE FOR INCREASING THE SENSITIVITY OF TESTS FOR SPECIFIC TREPONEMAL ANTIBODY

BARBARA J. ROSENAU, SC.D., AND JOHN F. KENT, PH.D.*
WASHINGTON, D. C.

WITH THE ASSISTANCE OF ROBERT W. SANDERS AND LOIS A. SIMONTON, B.S.

THE percentage of *Treponema pallidum* specifically immobilized[1] or rendered methylene blue resistant[2] by antibody and complement (C′) increases with time, but deterioration of C′ at the optimal reaction temperature (35° C.) has limited incubation generally to 18 hours.[3-5] Longer periods have been used[6] with resultant increase in sensitivity for treponemal antibody, but in our experience the advantage in sensitivity of such techniques was too often negated by losses in C′ activity. Methods of resolving the problem were explored, and a simple procedure based on supplemental additions of C′ was developed and evaluated.

MATERIALS AND METHODS

The standard 18-hour treponemal immobilization and methylene blue tests were conducted according to established procedure.[2, 4] For the 40-hour tests anaerobiasis was interrupted at 18 hours, and supplements (0.05 ml.) of active and heat-inactivated guinea pig C′ were added to tests and controls, respectively. Anaerobiasis was quickly re-established, and incubation was continued to 40 hours. The syphilitic rabbit sera were from animals infected experimentally with the Nichols stain of *T. pallidum*. The human sera were obtained from (1) syphilitic patients with diagnoses confirmed either by dark-field demonstration of treponemes or by specific serologic tests,[2, 4] (2) patients with nontreponemal infections or noninfectious disorders, who were seronegative in specific tests[2, 4] for syphilis, and (3) healthy seronegative individuals. All were stored at −20° C. Just prior to testing, the required volume of thawed serum was heated 30 minutes in the water bath; rabbit serum was heated at 60° C., human serum at 56° C.

RESULTS AND DISCUSSION

Titrations of syphilitic rabbit sera using the 18-hour and 40-hour procedures are represented in Table I. Prolonged incubation mediated by supplemental additions of C′ more than doubled the percentage of treponemes specifically immobilized at a given serum dilution; there was a corresponding increase in the percentage of organisms rendered specifically resistant to methylene blue. On the basis of these findings the studies were extended to human sera. Table II summarizes the results obtained in qualitative 18-hour (TP_{18})

From the Department of Serology, Walter Reed Army Institute of Research, Washington, D. C.

Presented in part at the Symposium on Recent Advances in the Study of Venereal Diseases, U. S. Department of Health, Education, and Welfare, April 25, 1957, Washington, D. C.

Received for publication Sept. 17, 1957.

*Division of Laboratories and Research, N. Y. State Department of Health, New Scotland Avenue, Albany, N. Y.

TABLE I. INFLUENCE OF PROLONGED INCUBATION MEDIATED BY SUPPLEMENTAL ADDITIONS OF COMPLEMENT ON THE TREPONEMAL IMMOBILIZATION REACTION

SYPHILITIC RABBIT SERUM DILUTED 1:	SPECIFIC IMMOBILIZATION (%) AFTER THE GIVEN TIME (HR.)*			
	SERUM 72452		SERUM 82252	
	18	40	18	40
8	100	100	88	100
16	93	96	64	100
32	47	93	20	69
64	9	80	0	33
128	4	27	0	22

*Comparable results were obtained in tests for specific resistance to methylene blue.

TABLE II. RELATIVE SENSITIVITY AND SPECIFICITY OF THE 18-HOUR (TP_{18}) AND 40-HOUR (TP_{40}) PROCEDURES AS DETERMINED IN QUALITATIVE TESTS OF HUMAN SERA

DIAGNOSIS	PERSONS TESTED	RESULT* USING THE GIVEN PROCEDURE				
		TP_{18}		TP_{40}		
		—	WR	—	WR	R
Syphilis†						
primary	19	17	2	9	3	7
secondary	5	1	4			5
early treated	7	6	1	2	1	4
congenital	1	1				1
neurological	3	3		1	1	1
cardiovascular	4	4		1		3
latent (probable)	5	4	1			5
Totals	44	36	8	13	5	26
Nontreponemal infections‡	10	10		9	1	
Noninfectious disorders‡	25	25		25		
Healthy	32	32		32		

*Designated nonreactive (–), weakly reactive (wr), or reactive (R). Identical results were observed in treponemal immobilization (TPI) and methylene blue (TPMB) tests, and the two are represented as TP.

†Selected patients found nonreactive or weakly reactive in TP_{18} tests.

‡All patients reactive in tests[5, 9] using lipoidal antigens.

and 40-hour (TP_{40}) tests; the treponemal immobilization and methylene blue reactions were identical, and are represented as one (TP). The syphilitic sera used in evaluating sensitivity were from a selected group of 44 patients found to be nonreactive or weakly reactive in standard 18-hour tests. Of the 36 patients in the nonreactive (–) group, 13 remained nonreactive (–), 5 proved weakly reactive (wr), and 18 proved reactive (R) in TP_{40} tests. The 8 patients that showed weak TP_{18} reactions were reactive in TP_{40} tests. There was thus a significant increase in sensitivity for specific treponemal antibody. The findings in primary and secondary syphilis were of special interest, the experience indicating that reported failures[1-3, 5, 7] to demonstrate the antibody in early infections may have been due in part to the use of relatively insensitive techniques. The evaluation of specificity was based principally on a group of 35 patients with nontreponemal infections or noninfectious disorders, who reacted serologically with lipoidal antigens[5, 9]; all were seronegative in standard TP_{18} tests. One of the group, a patient recovering from viral hepatitis, showed evidence of a reaction (wr) in TP_{40} tests, but the remainder were seronegative. No TP_{40} reactions were observed in a study of 32 healthy individuals. Further evaluation of the procedure is essential. However, its superior sensitivity

should make it useful in detecting persons with latent infections, and in differentiating them from individuals whose sera react adventitiously with lipoidal antigens.

SUMMARY

Prolonged incubation (40 hours) mediated by supplemental additions of complement considerably increased the sensitivity of treponemal immobilization (TPI) and methylene blue (TPMB) tests for syphilis; there was no significant loss in specificity.

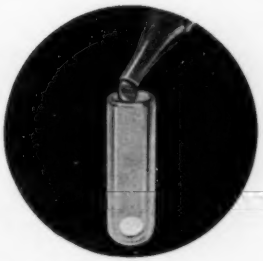
REFERENCES

1. Nelson, R. A., and Mayer, M. M.: Immobilization of Treponema Pallidum In Vitro by Antibody Produced in Syphilitic Infection, J. Exper. Med. **89:** 369-393, 1949.
2. Rosenau, B. J., and Kent, J. F.: Resistance of Specifically-Sensitized Treponema Pallidum to Methylene Blue Stain, Proc. Soc. Exper. Biol. & Med. **91:** 579-582, 1956.
3. Magnuson, H. J., and Thompson, F. A.: Treponemal Immobilization Test of Normal and Syphilitic Serums, J. Ven. Dis. Inform. **30:** 309-320, 1949.
4. Nelson, R. A., and Diesendruck, J. A.: Studies on Treponemal Immobilizing Antibodies in Syphilis. I. Techniques of Measurement and Factors Influencing Immobilization, J. Immunol. **66:** 667-685, 1951.
5. Thompson, F. A., and Magnuson, H. J.: Studies on Increasing the Sensitivity of the Treponemal Immobilization Test for Syphilis, Am. J. Syph., Gonor. & Ven. Dis. **35:** 21-34, 1951.
6. Nielson, H. A.: Clinical Value of the TPI Test, Acta dermat.-venereol. **34:** 102-111, 1954.
7. Nelson, R. A., Zheutlin, H. E. C., Diesendruck, J. A., and Austin, P. G. M.: Studies on Treponemal Immobilizing Antibodies in Syphilis. II. Incidence in Serum and Cerebrospinal Fluid in Human Beings and Absence in "Biologic False Positive" Reactors, Am. J. Syph. Gonor. & Ven. Dis. **34:** 102-121, 1950.
8. Harris, A., Rosenberg, A. A., and Riedel, L. M.: A Microflocculation Test for Syphilis Using Cardiolipin Antigen. Preliminary Report, J. Ven. Dis. Inform. **27:** 169-174, 1946.
9. Kent, J. F., Boyd, H. M., and Sanders, R. W.: Cardiolipin Antigen in the Kolmer Complement Fixation Test for Syphilis, Bull. U. S. Army M. Dept. **8:** 284-293, 1948.

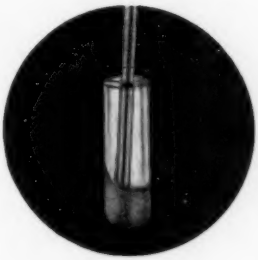
IT'S THIS FAST!

IT'S THIS EASY!

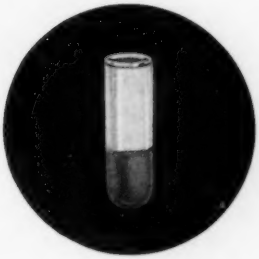
IT'S ACCURATE!



1. Add 4 drops serum or plasma to one PHOSPHATAB tablet in special tube.

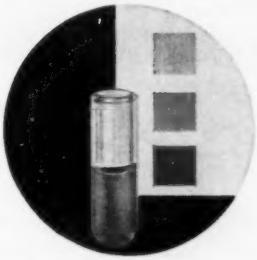


2. Crush tablet.



3. Let stand at room temperature 12-30 minutes. (time from chart)

4. Add 1 drop of color developer: mix.



5. Compare color.

IT'S HERE! STAT ASSURANCE in **your alkaline phosphatase testing.**

First control available over routine tests.

Time-saving, work-saving gauge for proper dilution for longer work up.

Rapid tool for sorting out normal sera.

Quick, clinically-significant way to do essential last-minute "stats".

TM

PHOSPHATABS

(alkaline) *with Teswells*

ORDER NOW

from your laboratory distributor

Laboratory Supply Division

WARNER-CHILCOTT

MORRIS PLAINS, N. J.

NOW he is like a tightly wrapped bud. But, petal by petal, you will help the future unfold in your child. Sobering idea, isn't it? Makes a thoughtful parent resolve to begin saving now for the richer future a good education can provide. And what better way to save than with U. S. Savings Bonds? Where nothing can harm that education fund. Where saved dollars earn 3¼% interest when Bonds are held to maturity. And where saving is surer—because you can buy Bonds regularly where you bank or automatically through the Payroll Savings Plan where you work. Why don't you start your Bond program today? Make life more secure for someone you love.

The U. S. Government does not pay for this advertisement. It is donated by this publication in cooperation with the Advertising Council and the Magazine Publishers Association.

Specializing

ENDOCRINE ASSAYS

17-Ketosteroids

Beta and Androgen Fractions
Protein-Bound Iodine • Pregnanediol
Pregnanetriol • Gonadotropins
Corticoids • Estrogens

TOXICOLOGY

Lead • Arsenic • Barbiturates

MISCELLANEOUS

Transaminase • Sodium • Potassium
Antistreptolysin • Proteins • Lipids

CONSERVATIVE FEES

Write for monthly "Bulletin of Laboratory Medicine" and fee schedule
Airmail service anywhere in the U.S.A.

Biochemical Procedures
8350 WILSHIRE BOULEVARD, BEVERLY HILLS, CALIFORNIA

Changing Your Address?

When you move, please—

(1) Notify us to change your address—allow us six weeks to make the change.

(2) Mention the name of this Journal. (We publish twelve periodicals.)

(3) Give us your old address. If possible, return the addressed portion of the envelope in which we sent your last copy.

(4) Give us your new address—complete—including the Postal zone number.

(5) Please print your name and address.

Thank You!

Circulation Department, The C. V. Mosby Company, Publishers, 3207 Washington Blvd., St. Louis 3, Mo.

KLETT SCIENTIFIC PRODUCTS

Klett-Summerson Photoelectric Colorimeters, Colorimeters, Nephelometers, Fluorimeters, Bio-Colorimeters, Comparators, Electrophoresis Apparatus, Glass Standards, Glass Cells, Klett Reagents

THE KLETT-SUMMERSON
PHOTOELECTRIC COLORIMETER

Developed with the requirements of routine colorimetric analysis particularly in mind, but has found application in a variety of unusual analytical procedures as well. No logarithm tables or "calibration curves" on semilogarithmic paper required. The concentration of unknown is obtained directly from the scale reading by simple calculation.

$205

KLETT BIO-COLORIMETER

The visual colorimeter illustrated presents a number of outstanding features making it ideal for clinical or research work. Built on the most modern mechanical and optical principles it is widely accepted for its accuracy and durability.

$165

Klett **MANUFACTURING CO.**
179 EAST 87th STREET, NEW YORK, N. Y.

THE ENCOURAGEMENT OF PROGRESS

The American Cancer Society's annual Spring Crusade is the climax of its year-round attack on cancer through research, professional and lay education, and service to the stricken. A study of the cancer scoreboard indicates that steady progress is being made. More and more lives are being saved. Progress encourages more progress.

Earlier diagnosis, new methods of treatment and a greater public awareness have contributed to this progress. It is often said that the life of the cancer patient is in the hands of the first physician he consults. The Society, therefore, conducts a broad professional education program, making available to doctors, through literature, films, exhibits, and other materials, information on the latest advances in detection, diagnosis and treatment.

As the Society aids the doctor, so does its large corps of volunteers aid the cancer patient with dressings, transportation, home care, medication and a host of other vitally needed services.

For the past two years, the theme of the Society's annual Crusade has been "Fight Cancer with a Checkup and a Check." That Americans everywhere are learning the value of the annual health checkup in the fight against cancer, is evidenced by the fact that doctors report they are now seeing more cancer in its earliest stages than ever before.

That American men and women have a personal stake in the program of the American Cancer Society is demonstrated by the public's generous support of the Crusade. This year the goal is $30,000,000 and we are confident that our people will meet the challenge . . . will "fight cancer with a checkup and a check" in the encouragement of further progress.

Lowell T. Coggeshall, M.D., *President*
American Cancer Society

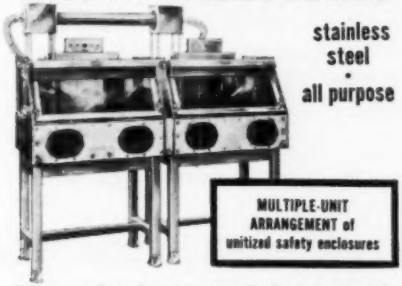
There once was a lab tech named Jess
Whose typing sometimes caused distress
Till he used our fine serum
Now no one need fear him
He works with aplomb and finesse.

You'll like the blood typing serums by

Knickerbocker

300 West 43rd Street, New York City

UNITIZED
SAFETY ENCLOSURES



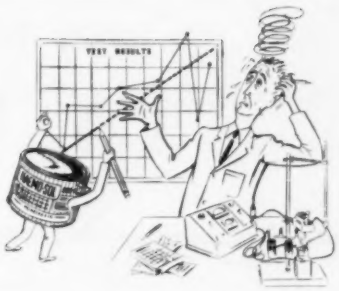
stainless steel • all purpose

Mass-produced, with the limited budget in mind, these protective enclosures offer modular adaptability. Single or multiple units, with various adapter plates, air locks, accessories, to fit your need. Integral 5-inch cup sink in bottom tray of each unit. Many other features now in use in laboratories of AEC and Chemical Corps. Write for illustrated folder describing this and 21 other kinds of enclosures. S. Blickman, Inc., 9204 Gregory Avenue, Weehawken, New Jersey.

BLICKMAN SAFETY ENCLOSURES

Look for this symbol of quality Blickman Built

See us at the Biology Show; Trade/Convention Center, Philadelphia, April 14-18



TOO MANY VARIABLES?

It's time to draw a line. Straighten out your cleaning problems with

HAEMO-SOL

There's nothing like Haemo-Sol's unique cleansing power and positive rinsing . . . it's completely safe! No etching! No corroding of metal parts! Immediate Haemo-Sol bath for valuable volumetric and optical equipment prevents soil etching!

Haemo-Sol guarantees clean laboratory glassware and apparatus—

- removes the full range of laboratory soils
- effectively digests protenoid materials . . . other types of polymeric materials
- assures free draining pipets . . . burets
- gives sparkling clear surfaces for quartz and glass absorption cells
- provides chemically clean reaction and titration flasks
- leaves the clean surfaces that are a must for the smooth operation of fractionating columns and other pieces of laboratory equipment.

And, just as important as its unique cleaning power, is Haemo-Sol's *high solubility* and powerful solubilizing action. Haemo-Sol washed glassware *rinses completely clean . . . nothing remains behind* but a chemically clean, free draining glass surface.

Write TODAY for Sample and Literature

Distributed by

MEINECKE & CO., INC.
Varick Street • New York 14

Overnight service from our South Carolina branch, 419 Gadsen St., Columbia, S. C.

New Third (1957) Edition!

PATHOLOGY

Edited by

W. A. D. ANDERSON, Professor of Pathology and Chairman of the Department of Pathology, University of Miami School of Medicine; Director of the Pathology Laboratories, Jackson Memorial Hospital, Miami, Fla.

Third (1957) Edition. 1402 pages, 1294 illustrations, 11 color plates. PRICE, $16.00.

This outstanding work on pathology has in the space of a few years won a fond place in the minds and hearts of all ranks of medicine. As in the literature of surgery and internal medicine where compilations by a group of well recognized experts have attained considerable lasting recognition, this volume similarly brings to the student of pathology the advantage of multiple specialist opinions. The list of contributors is indeed a galaxy selected carefully from the American scene. Their articles are uniformly clearly written, beautifully illustrated and possessed of fine craftsmanship.

Essential facts about any pathological process can be found in the portions of the text in larger face type, and more detailed material is presented in smaller type. The text is terse, definite, and understandable.

No effort has been spared to bring this edition thoroughly up to date. Many sections have been completely rewritten, others rearranged and rewritten to incorporate the newer advances in pathology. The bibliographies, always a feature because of their extensiveness, have naturally been revised and expanded.

Reviewing the last edition "American Practitioner and Digest of Treatment" had this to say: "This book remains the most complete and informative single volume on the topic of pathology available today."

Order today from

THE C. V. MOSBY CO.

3207 Washington Blvd.
St. Louis 3, Mo.

Date _____

THE C. V. MOSBY COMPANY
3207 Washington Blvd.
St. Louis 3, Missouri

Gentlemen: Send me Anderson "PATHOLOGY"—New Third (1957) Edition. Price, $16.00.

☐ Attached is my check. ☐ Charge my account.

Dr. _____

Street _____

City _____ Zone ____ State _____

LAB-4-58

NEW SANBORN SYSTEMS AND INSTRUMENTS

—on display at the current conventions*

PHOTOGRAPHIC RECORDING SYSTEM

. . . up to 8 channels

Completely new, highly flexible *"550M Poly-Beam"* provides up to 8 channels for recording — and up to 4 channels for monitoring — in single, standard size mobile cabinet. Recorder uses 15 cm. wide paper, with entire chart width available to each galvanometer beam; can be equipped originally for 4 channels, later easily expanded to 6 or 8 channels by adding coil-and-mirror galvanometer inserts. Optional *Monitoring Unit* incorporates an oscilloscope and electronic switch, to permit instantaneous visual presentation of as many as four waveforms simultaneously, and a vector timer for display of vector loops on the oscilloscope. Also available are new, miniaturized interchangeable plug-in *Preamplifiers*, for recording numerous phenomena such as an ECG, EEG, fetal ECG, phonocardiogram, pressure, temperature, etc.

DIRECT WRITING SYSTEM . . . 6- & 8-channel

New "350M" system also features single cabinet packaging, interchangeable Preamplifiers, optional Monitoring Unit. Flush front recorder has 8" of visible record, electrical push-button chart speed control with contacts for remote control, extended frequency response to 120 cps, fully transistorized Power Amplifiers . . . and many other operating and performance advantages.

TRANSDUCERS

. . . temperature and pressure

The Sanborn exhibit will also show for the first time a new *resistance thermometer* with five probes, providing ten ranges between 0° and 100°C, sensitivity of 0.2°C/cm deflection, and rapid response . . . as well as new wider range pressure transducers, including a type for use in gas measurements.

"FAMILIAR" SANBORN INSTRUMENTS

displayed and demonstrated will include such equipment as the Sanborn-Ampex Heart Sound Tape Recorder/Player . . . Model 51 Viso and Model 300 Visette electrocardiographs . . . Twin-Beam 2-channel system . . . Viso-Scope oscilloscope . . . and others.

You are cordially invited to stop by to see these new Sanborn research instruments — shown for the first time at these conventions. Your questions and comments will — as always — be most welcome!*

*FED. AMER. SOC. EXP. BIOLOGY Philadelphia—April 14-18 Booth 14 & 15

AMER. COL. PHYSICIANS Atlantic City—April 28-May 2 Booth 204 & 206

AMER. SOC. CLINICAL INVESTIG. Atlantic City—May 4-6 Booth 3 & 4

SANBORN COMPANY

MEDICAL DIVISION

175 Wyman St., Waltham 54, Mass.

You can do without ballet
And the drama

You can do without westerns
Or Cinerama

You can do without jazz
And opera, too

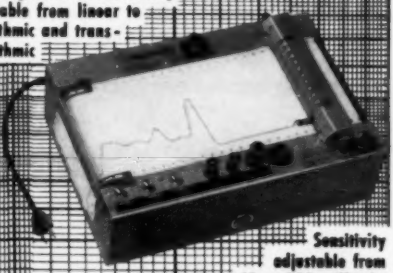
You can do without PROTROL*
. . . but why should you?

Knickerbocker
300 West 43rd Street, New York City

*T.M. Reg. U.S. Pat. Off. PROTROL, now the most successful prothrombin control reference standard in the world. You'll love it because it's easy-to-use, pure and human — your *true* prothrombin control — not an animal plasma.

VARICORD
VARIABLE-RESPONSE RECORDER
for densitometry in paper electrophoresis

Recording function electrically adjustable from linear to logarithmic and trans-logarithmic



Sensitivity adjustable from 10 to 200 millivolts

Designed specifically to be used with scanning densitometers for correctly-compensated quantitative evaluation of electrophoretic patterns on filter paper

Write for Bulletin #1100

Also: Densitometers pH Meters Colorimeters Fluorescence Meters Electronic Photometers Multiplier Photometers

PHOTOVOLT CORP.
95 Madison Ave. New York 16, N. Y.

FOR MEN AND WOMEN!

A WORTHWHILE CAREER IN LABORATORY TECHNIQUE

THE GRADWOHL SCHOOL OF LABORATORY TECHNIQUE is an ethical school, manned by competent ethical physicians and technologists and enjoys a high rating among the medical profession. . . . Graduates placed in desirable positions since 1920. Gradwohl graduates are recognized as expert technicians.

Course includes—Clinical Microscopy, Hematology and Blood Grouping; Serology; Applied Bacteriology; Basal Metabolism; Blood Chemistry; Electrocardiography; Parasitology; Tissue Cutting and Staining.

ENROLL NOW for priority. 12-month course; 6-month internship. Classes start Jan., March, July, Sept., Oct.

37th Successful Year

GRADWOHL SCHOOL OF LABORATORY TECHNIQUE

Under the Personal Supervision of
R. B. H. Gradwohl, M. D., Sc. D., Director
3514 Lucas Ave. St. Louis 3, Mo.

VIRUS AND RICKETTSIA — Complement Fixation

LIPID AND ENZYME CHEMISTRY

SPECIAL CHEMICAL PROCEDURES—P.B.I.—Mineral—Organic

72 to 96 hour service anywhere in the USA—Fee Schedules and Information on Request

Clinical Laboratory of San Bernardino

955 "D" Street, P. O. Box 329, San Bernardino, California

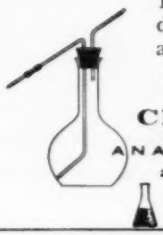
MEDICAL TECHNOLOGIST-CHEMISTRY

Masters in Chemistry or Bio-Chemistry with experience in Clinical Chemistry. Salary range begins at the rate of $5880 per annum, depending on qualifications. Personnel policy includes annual increments, 40 hour week, 7 paid holidays, and 4 weeks paid vacation.

Reply to: WERNER A LAQUEUR, M.D., Director of Laboratories
Beckley Memorial Hospital, Beckley, West Virginia

REFERRED BIOCHEMICAL ANALYSES

Electrophoresis for Protein Fractionation, Lipoprotein Fractionation, Abnormal Hemoglobins; **Catecholamine** determinations for adrenaline and noradrenaline; **Transaminase** levels; **Hormone** Assays; **Protein-Bound Iodine** determinations; **Toxicological** Assays; Antistreptolysin-O Titers; and other difficult and time-consuming tests.



Services available nationally by Air Mail. Shipping containers with vials available on request

Write for: List of Services, Literature, and Containers

CHEM-TECH LABORATORIES

ANALYTICAL - CLINICAL - RESEARCH

2364-2384 SOUTH ROBERTSON BLVD. BEVERLY HILLS, CALIFORNIA

S. I. DULKIN, PH.D. OTTO E. LOBSTEIN, PH.D

Nessler Compound Paragon

(Dry Granules)

for the simplest method of preparing Nessler Solution which will not be affected by the amount of carbonates ordinarily present in sodium hydroxide. The granules and the resulting solution will remain stable for years.

$1.90 per bottle (68 grams) sufficient for one liter of Nessler Solution, which is made by simply dissolving the contents of bottle in 10 per cent sodium hydroxide solution.

Convince yourself by ordering Nessler Compound Paragon. Available for immediate delivery.

Benedict Compound Paragon

Qualitative—Quantitative

(Dry Granules)

You can now have perfect Benedict Solutions (qualitative or quantitative) for the determination of sugar in urine by simply dissolving the granules in water.

Packed in two convenient sizes:

QUALITATIVE	QUANTITATIVE
P501—for 1 liter	P611—for 1 liter
P520—for 1 gallon	P631—for 1 gallon

Priced so that each qualitative test costs ⅙ of a cent, when purchased in lots of 12 gallons (1 case); slightly higher when purchased in smaller quantities.

Efficient ***Convenient*** ***Economical***

Manufactured exclusively by

PARAGON C. & C. CO. INC., 2540 Belmont Avenue, New York 58, New York

(See also pages 41, 42)

LIVE FROGS for PREGNANCY DIAGNOSIS

Rana pipiens

Year 'round supply of selected large, healthy, male frogs for latest pregnancy test (99% accuracy). Cost negligible. Negative test animals can be used repeatedly. **Results in less than two hours.** Prompt shipment anywhere, by air to distant points. **Prices include screened shipping crates and boxes.**

Frogs. With complete directions for use and care. Dozen	**7.50**
Laboratory Tank with Cover	**7.00**
Isolation Jar, 5" x 7" with weighted metal cover	**3.50**
Frog pregnancy test kit, complete, 14 items including 12 live frogs	**35.00**

Est. 1919

NEW YORK SCIENTIFIC SUPPLY CO. - - - - 28 West 30th St., New York 1, N. Y.

Headquarters for Live Frogs

"Our 43rd Year—Time Proved Dependability"

RABBITS & GUINEA PIGS, ALBINO RATS & MICE

RESEARCH SUPPLY CO.

2436 West York St. **Philadelphia 32, Pa.**

DAN ROLFSMEYER CO.

ALBINO RATS **SWISS (ALBINO) MICE**

Price List on Request

Route 3, Syene Road, Madison 5, Wis. **PHONE ALpine 6-6149**

Live Male Rana Pipien Frogs, Direct from the Farm

Be sure of year round supply, at lowest prices and fastest service available. Orders delivered within 36 hours after we receive them.

Male Rana Clamitan Frogs and Buffo Americanus Toads available during summer months.

Male Buffo Marinus Toads available, the year round.

Animals guaranteed free of disease and to arrive in perfect condition.

Laboratory Manual 85 pages with illustrations. A system of pregnancy testing using Male Toads and Frogs, by Edward H. Hon, M.D., of Yale University.

Also reagents used in conducting pregnancy tests.

For Information and prices, Phone or write:

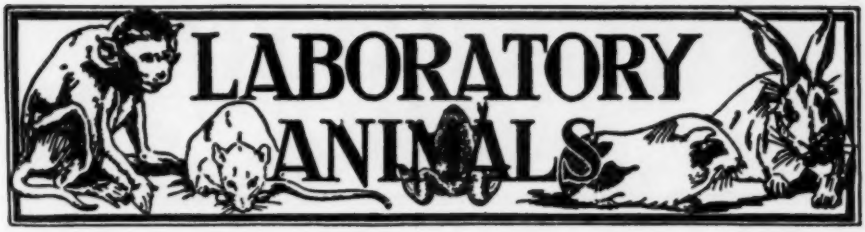
J. R. SCHETTLE FROG FARM, INC. Stillwater, Minnesota

WHITE MICE GUINEA PIGS RABBITS

When full grown our Albino mice go 35 to 45 gms.

Send for price list. References furnished on request.

J. E. STOCKER · Ramsey, N. J. · Phone: DAvis 7-1064



(See also pages 40, 42)

SINCE 1945

XENOPUS LAEVIS FROGS
exclusively

X-L FEMALE FROG PREGNANCY TEST

NO SEASONAL VARIATIONS

Combines highest accuracy — economy — speed. Test so simple requires minimum of technician's time using either urine concentrate or blood serum. No microscope work required or surgery of the animal. Write for literature. Animal cost per test 10¢ to 15¢.

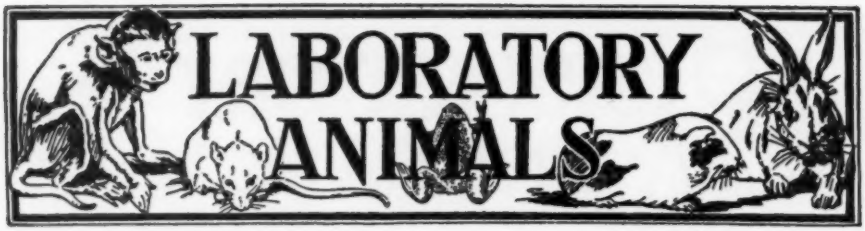
JAY E. COOK, Importer
Cockeysville, Maryland, U.S.A.

TACONIC FARMS, Inc.
Germantown, New York
SWISS MICE *Booklet Sent on Request*

ZEIMET BIO-FARMS
DOCILE **GUINEA PIGS** *DEPENDABLE*
Route 4, Box 372 CHerry 9-4515 Madison 4, Wis.

HAMILTON LABORATORY ANIMALS, INC.
Specialist in breeding of HLA Albino Rats and HLA Albino Swiss Mice. Prompt and efficient service. Dependable animals.
Office: 200 Brotherhood Bldg., 1015 Vine St., Cincinnati 2, Ohio

RABBITS **GUINEA PIGS**
Developed and housed to insure best results in all types of tests
Continuous supply and prompt shipment FOB Indianapolis
PAUL HAMM RABBITRY R.R. No. 4 Phone 530-W3 Greenwood, Ind.



(See also pages 40, 41)

HAMSTERS **GUINEA PIGS** **RABBITS** **WHITE MICE AND RATS**

Satisfactorily shipped 1500 miles and more — Large supply

For prices and information, write, wire, or phone

HILLTOP CAVIARY

Phone 286 Established 1928 Scottdale, Pa. (near Pittsburgh)

LABORATORY ANIMALS

We are specializing in an excellent strain of Albino Guinea Pigs—heavy in Hartley Blood Lines. Highly acclaimed by all users. Also Webster Swiss Albino Mice and Wistar Albino Rats.

Dogs	Rats	Rabbits
Cats	Pigeons	Hamsters
Mice	Poultry	Guinea Pigs

JOHN C. LANDIS, Hagerstown, Maryland

NATIONAL LABORATORY ANIMAL CO.

Reliable Animals for Significant Results

RABBITS—GUINEA PIGS—WHITE RATS—WHITE MICE

Prompt Shipment from Pittsburgh, Pa.

RD, Mars, Pa. **Phone—NAtional 5-1955**

• STANDARDIZED • • DEPENDABLE • **RATS** WISTAR (ALBINO) LONG-EVANS (HOODED)

BRED FOR RESEARCH WORK BY RESEARCH WORKERS

PACIFIC ANIMAL FARMS 14516 So. Budlong Ave. GARDENA, CALIF.

(An affiliate of Pacific Biological Laboratories)

LABORATORY ANIMALS

since 1929

Albino Swiss-W Mice
Albino Rats from Wistar Strain
Albino Guinea Pigs
Albino NZ Rabbits
Hamsters

ALBINO FARMS

P. O. Box 331
RED BANK, NEW JERSEY

MICE
RATS
RABBITS
GUINEA PIGS

CHERRY HILL FARMS

P.O. BOX 840, CAMDEN 1, N. J.

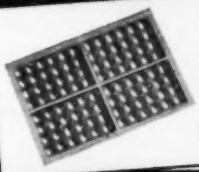
FOR THOROUGH CLEANING
of Laboratory Glassware and Equipment

NON-TOXIC FOR TISSUE CULTURE

7X® for Hand Washing

7X® o-matic for Mechanical and Automatic Washers

DISPOSO-TRAY®



for VIRUS TITRATIONS

Use as Test Tubes and Culture Tubes. So Low in Price You Can Use Them Once and Throw Them Away!

Sold by Dealers the World Over

LINBRO CHEMICAL CO., INC.
681 DIXWELL AVE., NEW HAVEN 11, CONN

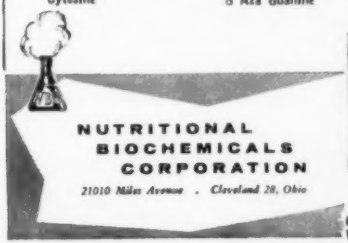
Searching for One Dependable Source for All Your Research Biochemical Needs

NUTRITIONAL BIOCHEMICALS CORPORATION offers you quality that merits your complete confidence . . . service you can always rely upon . . . plus the economy of lowest possible prices.

A COMPLETE SELECTION OF MORE THAN 200 NUCLEOPROTEINS and DERIVATIVES

Typical Derivatives

Adenosine Triphosphate	6 Mercaptopurine
Cytidine	Uridylic Acid
Cozymase	Uridine
Coenzyme I, II, A	2, 6 Diamino Purine Sulfate
Cytosine	8 Aza Guanine



NUTRITIONAL BIOCHEMICALS CORPORATION
21010 Miles Avenue . Cleveland 28, Ohio

Write for New Catalog Feb. 1958 Over 2100 Items Write Dept. 109

Here's an Rx every M.D. will O.K.!

Veeder-Root Hand Tallies for Speedy Blood Count

Doctors, lab technicians . . . everyone who has used a Veeder-Root Hand Tally or seen one operate, knows how much time is saved in taking differential or red and white count. Cupped comfortably and securely in either hand, other hand remains free for phone, slides, microscope or pencil work. Slightest pressure of lever registers each count. To reset, just turn knob to zero. Once you've tried a Veeder-Root Hand Tally, you'll wonder how you ever worked without one!

QUICK FACTS — *Single counter* counts up to 10,000. White figures, black background. ($8.65 each.)

DOUBLE COUNTER — Figures on one unit, white on black, other unit, red on white. Each unit counts up to 10,000. ($19.60 each.)

If not available from your Medical Supply House, use coupon below.

Veeder-Root

"The Name That Counts"

World's Largest Makers of Counting Devices

VEEDER-ROOT INCORPORATED
HARTFORD 2, CONN.

Please send me descriptive folder of Veeder-Root Hand Tally Counters, together with current prices.

Name .....

Street .....

City ..... *State* .....

Please send me ..... ☐ Single ☐ Double Hand Tallies C.O.D.

JL-4-8

The Journal of Laboratory and Clinical Medicine

The Official Publication of the Central Society for Clinical Research

EDITOR
William D. Robinson, M.D.

ASSOCIATE EDITOR
William H. Beierwaltes, M.D.

University Hospital
University of Michigan Medical School
1313 Ann Street
Ann Arbor, Michigan

PUBLISHED BY THE C. V. MOSBY COMPANY, 3207 WASHINGTON BLVD.
ST. LOUIS 3, U. S. A.

Published monthly. Subscriptions may begin at any time.

Editorial Communications

The Journal of Laboratory and Clinical Medicine is published under the editorial direction of a Board of Editors appointed by the Central Society for Clinical Research. Manuscripts describing original observations in the broad fields of clinical investigation and clinical pathology are invited. Society affiliation will not prejudice or influence editorial decisions.

Manuscripts.—All correspondence relating to the publication of papers should be addressed to the editor. Manuscripts must be typewritten, double or triple spaced, on only one side of the paper. They should be organized under the following general headings: Introduction, Materials and Methods, Results, Discussion, and Summary. Academic degrees of authors should be given. Bibliographic references should include the titles of papers and should conform to the following style: Author, Title, Journal, Volume, Inclusive Pages, Year. Only one copy, the original, should be submitted.

Neither the editor nor the publisher accepts responsibility for the opinions of contributors, nor are they responsible for other than editorial statements.

Illustrations.—A reasonable number of halftone illustrations will be reproduced free of cost to the author, but special arrangements must be made with the editor for color plates, elaborate tables, or extra illustrations. Copy for zinc cuts (such as pen drawings and charts) should be drawn and lettered in India ink or black typewriter ribbon (when the typewriter is used). Only good glossy photographic prints or original drawings should be supplied for halftone work.

Reprints.—Reprints must be ordered directly from the publishers, The C. V. Mosby Company, 3207 Washington Boulevard, St. Louis 3, Mo., who will send their schedule of prices. Individual reprints of an article must be obtained through the author.

Business Communications

Business Communications.—All communications in regard to advertising, subscriptions, change of address, etc., should be addressed to the publishers, The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo.

Subscription Rates.—United States and its Possessions $13.00, Students $6.50; Canada, Latin-America, and Spain $13.50, Students $7.00; Other Countries $14.50, Students $8.00. Single copies, $2.00 postpaid. Remittances for subscriptions should be made by check, draft, post office or express money order, payable to this Journal.

Publication Order.—The monthly issues of this Journal form two semiannual volumes; the index is in the last issue of the volume—in the June and December issues.

Change of Address Notice.—Six weeks' notice is required to effect a change of address. Kindly give the exact name under which a subscription is entered, and the full form of both old and new address, including the post office zone number.

Advertisements.—Only articles of known scientific value will be given space. Forms close first day of the month preceding date of issue. Advertising rates and page sizes will be furnished on application.

Bound Volumes.—Publishers' Authorized Bindery Service, 5811 West Division Street, Chicago 51, Illinois, will quote prices for binding complete volumes in permanent buckram.

Increase the Range of Your Office Work

Improve the Accuracy of Your Diagnosis

Williamson

Practical Use of the OFFICE LABORATORY and X-RAY

(Including the Electrocardiograph)

1957
322 pages
6⅞" x 10"
illustrated
Price, $10.75.

A knowledge and understanding of the scope and limitations of your laboratory equipment and x-ray *and the proper interpretation of tests and films* are the keys to successful use of your equipment. With these keys you can increase the accuracy of your diagnosis and broaden the scope of your office practice.

In clear, understandable language, "Practical Use of the OFFICE LABORATORY and X-RAY" shows how you can utilize your equipment to maximum benefit—how you can make your laboratory and x-ray valued tools *to answer specific questions designed to confirm or deny your original findings* and not merely instruments to make "routine test reports."

Each procedure discussed can be done by a home-trained technician in any office with a minimum of basic equipment. Carefully-organized and well-illustrated, this practical guide discusses in detail each specific test, the equipment to be used, the technique, the interpretation of results and the errors most commonly made. The useful information you can gain from your office laboratory and x-ray is far more extensive than many physicians achieve with such equipment.

By PAUL WILLIAMSON, M.D.

Date_____

The C. V. MOSBY Company

3207 Washington Boulevard, St. Louis 3, Missouri

Gentlemen: Please send me Williamson, "PRACTICAL USE OF THE OFFICE LABORATORY AND X-RAY" priced at $10.75 on 10 day approval. I understand if I am not completely satisfied I can return the book within 10 days with no charge or obligation. If remittance is enclosed, publisher pays the mailing charge.

☐ Payment enclosed ☐ Charge my account

Dr. _____

Street _____

City _____ Zone _____ State _____

LAB-4-58

INDEX TO ADVERTISERS

Please mention "The Journal of Laboratory and Clinical Medicine" when writing to our advertisers—it identifies you

Albino Farms (Laboratory Animals) ... 42
Aloe Scientific, Div. of A. S. Aloe Company ... 15
American Cancer Society ... 34
American Electronics, Inc. ... 14
American Hospital Supply Corporation 9, 29
Ames Company, Inc. ... 10

Baker & Adamson ... 16
Beckley Memorial Hospital ... 39
Biochemical Procedures ... 33
Blickman, Inc., S. ... 35
Bristol-Myers Company ... Fourth Cover

Chem-Tech Laboratories ... 39
Cherry Hill Farms (Laboratory Animals) ... 42
Clay-Adams, Inc. ... 21
Clinical Laboratory of San Bernardino 39
Cook, Importer, Jay E. (Xenopus Laevis Frogs) ... 41
Corning Glass Works ... 17

Difco Laboratories ... 1

Eaton Laboratories ... 13

Farrand Optical Company, Inc. ... 23
Fisher Scientific ... 18

Gradwohl School of Laboratory Technique ... 38

Hamilton Laboratory Animals, Inc. ... 41
Hamm Rabbitry, Paul (Rabbits and Guinea Pigs) ... 41
Harshaw Scientific, Div. of The Harshaw Chemical Company ... 4
Hartman-Leddon Company ... 19
Hilltop Caviary (Laboratory Animals) 42
Hyland Laboratories ... 28

International Equipment Company ... 6

Kimble Glass Company ... 2, 3
Klett Manufacturing Company ... 33
Knickerbocker Biosales, Inc. ... 35, 38

Laboratory Animal Section ... 40, 41, 42
Landis, John C. (Laboratory Animals) 42
Leitz, Inc., E. ... 26
Linbro Chemical Company, Inc. ... 43

Meinecke & Company, Inc. ... 35

National Laboratory Animal Company (Laboratory Animals) ... 42
New York Scientific Supply Company (Frogs) ... 40
NRD Instrument Company, Div. of Nuclear Corporation of America, Inc. 24
Nuclear-Chicago Corporation ... 11
Nutritional Biochemicals Corporation ... 43

Ortho Pharmaceutical Corporation ... 5, 25

Pacific Animal Farms (Rats) ... 42
Paragon C. & C. Company, Inc. ... 12, 39
Photovolt Corporation ... 38

Research Supply Company (Laboratory Animals) ... 40
Roehr Products Company, Inc. ... 7
Rolfsmeyer Company, Dan (Rats and Mice) ... 40

Sanborn Company ... 37
Schettle Frog Farm, Inc., J. R. (Frogs) 40
Standard Scientific Supply Corporation 22
Stocker, J. E. (Laboratory Animals) ... 40

Taconic Farms, Inc. (Swiss Mice) ... 41
Technicon Company, The ... Third Cover
Thomas Company, Arthur H. ... 30

United States Savings Bonds ... 32

Veeder-Root Incorporated ... 43

Warner-Chilcott Laboratories ... 8, 27, 31

Zeimet Bio-Farms (Guinea Pigs) ... 41

While every precaution is taken to insure accuracy, we cannot guarantee against the possibility of an occasional change or omission in the preparation of this index.

automation finally brought to clinical chemistry

Already in hospitals and laboratories the country over, the Technicon Autoanalyzer is doing jobs like those listed below with an accuracy, reproducibility, and dependability, which has users standing back in awe. **The work goes faster,** up to 60 tests-per-hour, **better,** accuracy is repeatable to approximately 1%, **and cheaper,** completely automatic; no human intervention or supervision.

There's a brochure which explains the Autoanalyzer principle, detailing operation and methods.

clinical biochemical analyses

For the thousand-and-one substances for which biological fluids are analyzed . . . blood glucose, blood urea nitrogen, calcium, alkaline phosphatase, chlorides, etc. Reproducibility, approximately 1%. Elapsed time-per-test, as little as one minute.

pharmcodynamic research

Sampling by direct aspiration from circulatory (or other) system of a living organ, with continuous analysis and recording of phenomena like rate absorption or excretion of drugs, changes in concentration levels of blood urea nitrogen, blood glucose, etc.

completely automatic operation

Only human participation is initial setting up of test. Autoanalyzer then takes over; introduces correct proportions of materials and reagents, passes them through the train of analytical events — mixing, dialyzing, separation, heating, color-measuring, etc. — records test results as logarithmic or easy-reading linear graphs, or on digital read-out tapes. Selectable choice of 20, 40, or 60 separate tests-per-hour, depending on nature of test. Reproducibility, approximately 1%.

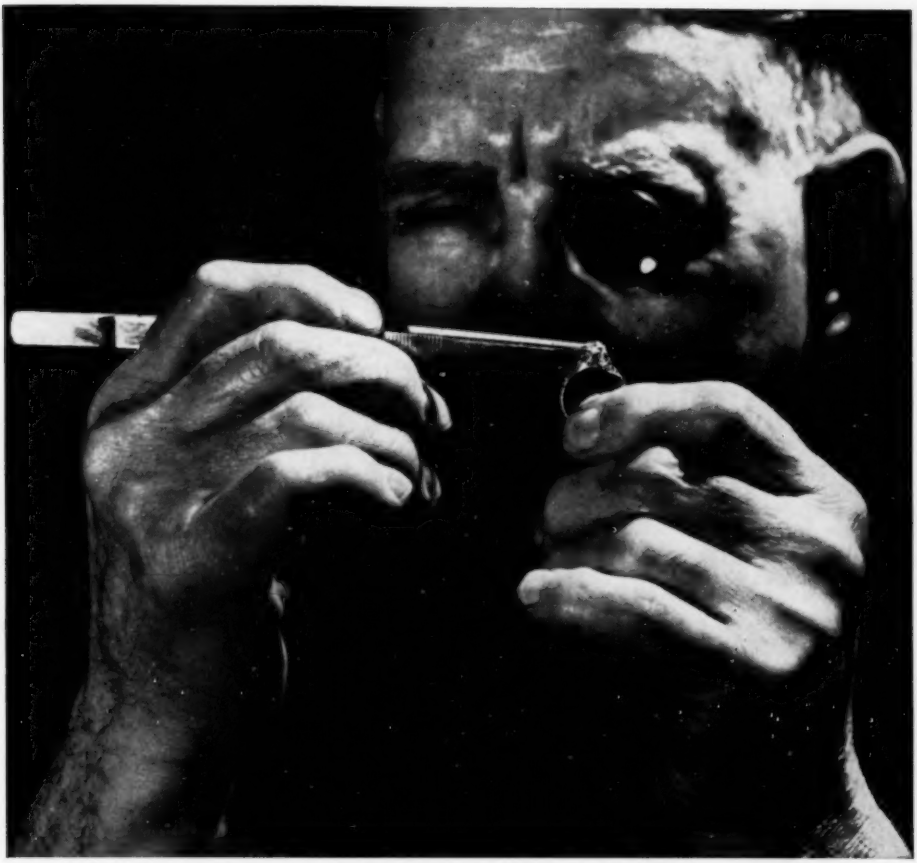
TECHNICON®

AutoAnalyzer

TRADE MARK

TECHNICON INSTRUMENTS CORPORATION

Chauncey • New York



release from pain and inflammation

with **BUFFERIN® IN ARTHRITIS**

salicylate benefits with minimal salicylate drawbacks

Rapid and prolonged relief—with less intolerance.

The analgesic and specific anti-inflammatory action of BUFFERIN helps reduce pain and joint edema—comfortably. BUFFERIN caused no gastric distress in 70 per cent of hospitalized arthritics with proved intolerance to aspirin. (Arthritics are at least 3 to 10 times as intolerant to straight aspirin as the general population.[1])

No sodium accumulation. Because BUFFERIN is sodium free, massive dosage for prolonged periods will not cause sodium accumulation or edema, even in cardiovascular cases.

Each *sodium-free* BUFFERIN tablet contains acetylsalicylic acid, 5 grains, and the antacids magnesium carbonate and aluminum glycinate.

Reference: 1. J.A.M.A. 158:386 (June 4) 1955.

ANOTHER FINE PRODUCT OF BRISTOL-MYERS

Bristol-Myers Company, 19 West 50 Street, New York 20, N. Y.